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The Royal Alexandra Hospital for Children

PROVISIONAL SPECIFICATION

Invention Title:

Regulation of cell surface proteins

The invention is described in the following statement:

*Regulation of cell surface proteins*Field of the Invention

- 5 The present invention relates to a method for regulating the insertion or retention of a protein in a cell surface membrane. The invention also relates to methods for the diagnosis and treatment or prevention of diseases caused by abnormal insertion or activity of a cell surface membrane protein. The invention further relates to methods of screening for compounds that regulate the transport of molecules into or out of a cell,
- 10 and for compounds that regulate the activity of cell surface proteins.

Background of the Invention

The establishment and maintenance of cell polarity is intrinsic to the function of an epithelial cell. The creation of these distinct functional domains relates to their role in providing a barrier and controlling ion and solute transport. Events leading to the development of this functional polarisation include cell-cell contact mediated by E-cadherin and cell-extracellular matrix adherence mediated by integrins. These spatial cues are communicated to the internal components of the cell via localised assembly of cytoskeletal and signalling complexes. This in turn directs reorganisation of the cell surface and the secretory system. The actin cytoskeleton, by virtue of its direct interaction with both integrin- and cadherin-containing complexes, plays a pivotal role in the establishment of epithelial cell polarity. Similarly, the actin filament system is responsible for targeting secretion in budding yeast. Thus, the actin cytoskeleton appears to play a role in the establishment of polarity in different phyla.

Polarised function of the actin cytoskeleton may go beyond specific interactions of actin filaments with integrin and cadherin containing complexes. There is increasing evidence that the isoform composition of actin filaments themselves can differ at different sites in a cell. In gastric parietal cells, the β and γ actin isoforms are differentially distributed in the cell with β actin located predominantly at the more metabolically active apical surface. Similar polarisation of β and γ actin is observed in adult neurons. Polarisation also extends to mRNA location where β , but not γ actin mRNA is specifically located at peripheral sites in the cell associated with motility such as lamellapodia and growth cones.

- There are a number of disease states in which alterations in epithelial cell polarity or defects in the process of targeted protein delivery are important features. In autosomal polycystic kidney disease, for example, aberrant expression of basolateral proteins on the apical membrane results in the production of fluid filled cysts. It has recently been
- 5 reported that 3 cytoskeletal binding proteins required for basolateral membrane organisation, E-cadherin, sec6 and sec8, are abnormally located within the diseased cells. This results in impaired delivery of proteins and lipids to the basolateral membrane (Charron *et al.*, 2000, *Journal of Cell Biology* 149, 111-124).
- 10 Cystic fibrosis is an autosomal recessive condition commonly due to the mutation ΔF508. This mutation results in abnormalities of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. In cystic fibrosis due to the ΔF508 mutation, the CFTR is abnormally folded and so is retained and degraded by the RER (Qu *et al.*, 1997, *Journal of Bioenergetics & Biomembranes* 29, 483-490; Brown and Breton, 2000, *Kidney International* 57, 816-824). In addition, CFTR with the ΔF508 mutation has a shorter half-life in the apical membrane (Heda *et al.*, 2001, *American Journal of Physiology - Cell Physiology* 280, C166-C174).
- 15 Alterations in the distribution of cytoskeletal proteins have also been observed in renal proximal tubule cells in response to ischaemia (Brown *et al.*, 1997, *American Journal of Physiology* 273, F1003-F1012). In rat kidneys, one hour of ischaemia and reperfusion was found to result in the relocation of the brush border proteins, villin and actin to the basolateral pole (Brown *et al.*, 1997, *American Journal of Physiology* 273, F1003-F1012). Partial restoration was seen twenty-four hours after reperfusion with full recovery occurring within five days. The authors postulated that the disassembly of the cortical actin cytoskeleton might allow changes in cell shape allowing surviving cells to cover areas of cell loss. In another study, it was found that ischaemia of renal proximal tubule cells resulted in dissociation of tropomyosin from F actin with the tropomyosin relocating to the distal aspects of the microvilli. These authors suggested that the relocation of tropomyosin allows a competing actin binding protein, actin depolymerising factor (ADF), to disrupt the apical microfilaments and thus apical microvilli.
- 20 Methods for the diagnosis and treatment of disease states caused by alterations in epithelial cell polarity or defects in the process of targeted protein delivery are highly desirable.

Summary of the Invention

- The present inventors have investigated the composition of actin microfilaments in gastrointestinal epithelial cells and their role in the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) into the apical membrane. This investigation has revealed a specific population of microfilaments containing tropomyosin (Tm) isoforms that are polarised in cell monolayers. Polarisation of this microfilament population occurs very rapidly in response to cell-cell and cell-substratum contact and involves the movement of intact microfilaments. Colocalisation of the TM isoforms and CFTR was observed in long-term cultures. A reduction in expression of the TM isoforms resulted in an increase in both CFTR surface expression and chloride efflux in response to cAMP stimulation. The results show that Tm isoforms mark an apical population of microfilaments that can regulate the insertion and/or retention of proteins into the plasma membrane.
- Accordingly, in a first aspect the present invention provides a method for regulating the insertion or retention of a protein in a cell surface membrane, the method comprising administering to the cell an agent that modulates tropomyosin expression, location or activity.
- In a further aspect the present invention provides a method for increasing the insertion or retention of a protein in the surface membrane of a cell, the method comprising administering to the cell a tropomyosin antagonist.
- In yet a further aspect the present invention provides a method for regulating the transport of molecules into or out of a cell, the method comprising administering to the cell an agent that modulates tropomyosin expression, location or activity.
- In yet a further aspect the present invention provides a method for increasing the transport of molecules into or out of a cell, the method comprising administering to the cell a tropomyosin antagonist.
- In one embodiment of the invention the molecules transported are selected from the group consisting of electrolytes, water, monosaccharides and ions.

In the context of the present invention, it is preferred that the tropomyosin is an isoform encoded by a gene selected from the non-limiting group consisting of TPM 1, TPM 2, TPM 3 and TPM 4. For example, the isoform may be selected from the group consisting of TM1, TM2, TM3, TM4, TM5, TM5a, TM5b, TM6, Tm5NM-1 -

- 5 Tm5NM-11, etc. In a preferred embodiment, the TM isoform is TM5a or TM5b.

A tropomyosin antagonist for use in the present invention may be selected from the group consisting of a peptide, an antibody directed against tropomyosin, a small organic molecule, an antisense compound directed against tropomyosin-encoding

- 10 mRNA, an anti-tropomyosin catalytic molecule such as a ribozyme or a DNAzyme, and a dsRNA or small interfering RNA (RNAi) molecule that targets tropomyosin expression. In a preferred embodiment the tropomyosin antagonist is an antisense compound directed against tropomyosin-encoding mRNA.

- 15 The protein inserted into the cell surface membrane is preferably selected from the group consisting of a transport protein, a channel, a receptor, a growth factor, an antigen, a signalling protein and a cell adhesion protein. The transport protein is preferably the cystic fibrosis transmembrane conductance regulator (CFTR).

- 20 In a further preferred embodiment of the present invention, the cell is a non-muscle and non-brain cell. In one preferred embodiment, the cell is an epithelial cell. Preferably, the epithelial cell is a gastrointestinal epithelial cell.

- 25 In yet a further aspect the present invention provides a method for assessing an individual's predisposition to a disease caused by the abnormal insertion, retention or activity of a cell surface membrane protein, the method comprising the step of determining the presence of a mutation in a tropomyosin gene of the individual.

- The mutation in the tropomyosin gene may be a point mutation (i.e. a single nucleotide polymorphism (SNP)), deletion and/or insertion. Such a mutation may be detected by isolating and sequencing DNA fragments from the tropomyosin gene or otherwise by isolating mRNA from the individual and synthesising DNA therefrom (e.g. by RT-PCR) for sequencing. Mutations may also be detected by hybridisation using discriminating oligonucleotide probes or by amplification procedures using 35 discriminating oligonucleotide primers.

In yet a further aspect the present invention provides a method for assessing an individual's predisposition to a disease caused by the abnormal insertion, retention or activity of a cell surface membrane protein, the method comprising analysing the polarised distribution of tropomyosin in the cells of the individual.

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If the distribution of a particular tropomyosin isoform differs in cells derived from the individual being tested from that observed in cells of a normal subject, this is indicative that the individual being tested has a predisposition to a disease caused by the abnormal insertion, retention or activity of a cell surface membrane protein.

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In yet a further aspect the present invention provides a method for the treatment or prevention of a disease in a subject caused by the abnormal insertion, retention or activity of a cell surface membrane protein, the method comprising administering to the subject an agent that modulates tropomyosin expression, location or activity.

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The disease caused by the abnormal insertion or activity of a cell surface membrane protein may be, for example, cystic fibrosis, multiple sclerosis, polycystic kidney disease, viral infection, bacterial infection, reperfusion injury, Menkes Disease, Wilson's Disease, diabetes, myotonic dystrophies, epilepsy or mood disorders such as depression, bipolar disorder or dysthymic disorder.

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In yet a further aspect the present invention provides a method of screening for a compound that regulates the transport of molecules into or out of a cell, the method comprising determining the activity of tropomyosin in the presence and absence of a

25 candidate compound, wherein altered tropomyosin activity in the presence of the compound indicates that the compound regulates the transport of molecules into or out of a cell.

In a preferred embodiment of this aspect reduced tropomyosin activity in the presence 30 of the compound indicates that the compound increases the transport of molecules into or out of a cell.

In yet a further aspect the present invention provides a method of screening for a compound that regulates the transport of molecules into or out of a cell, the method 35 comprising determining the expression levels of tropomyosin in the presence and absence of a candidate compound, wherein altered tropomyosin expression in the

presence of the compound indicates that the compound regulates the transport of molecules into or out of a cell.

- In a preferred embodiment of this aspect reduced tropomyosin expression in the
5 presence of the compound indicates that the compound increases the transport of molecules into or out of a cell.

- In yet a further aspect the present invention provides a method of screening for a compound that regulates the transport of molecules into or out of a cell, the method
10 comprising determining the ability of a candidate compound to modulate the binding of tropomyosin to one of its binding partners, wherein an altered level of binding of tropomyosin to its binding partner in the presence of the compound indicates that the compound regulates the transport of molecules into or out of a cell.
15 In a preferred embodiment of this aspect a reduced level of binding of tropomyosin to its binding partner in the presence of the compound indicates that the compound increases the transport of molecules into or out of a cell.

- In a further preferred embodiment of this aspect the tropomyosin binding partner is
20 actin.

- In yet a further aspect the present invention provides a method of screening for a compound that regulates the activity of a cell surface protein, the method comprising determining the activity of tropomyosin in the presence and absence of a candidate
25 compound, wherein altered tropomyosin activity in the presence of the compound indicates that the compound regulates the activity of a cell surface protein.

- In a preferred embodiment of this aspect reduced tropomyosin activity in the presence of the compound indicates that the compound increases the activity of a cell surface
30 protein.

- In yet a further aspect the present invention provides a method of screening for a compound that regulates the activity of a cell surface protein, the method comprising determining the expression levels of tropomyosin in the presence and absence of a candidate compound, wherein altered tropomyosin expression in the presence of the compound indicates that the compound regulates the activity of a cell surface protein.
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In a preferred embodiment of this aspect reduced tropomyosin expression in the presence of the compound indicates that the compound increases the activity of a cell surface protein.

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- In yet a further aspect the present invention provides a method of screening for a compound that regulates the activity of a cell surface protein, the method comprising determining the ability of a candidate compound to modulate the binding of tropomyosin to one of its binding partners, wherein an altered level of binding of 10 tropomyosin to its binding partner in the presence of the compound indicates that the compound regulates the activity of a cell surface protein.

In a preferred embodiment of this aspect a reduced level of binding of tropomyosin to its binding partner in the presence of the compound indicates that the compound 15 increases the activity of a cell surface protein.

In a further preferred embodiment of this aspect the tropomyosin binding partner is actin.

20 Brief Description of the Figures

Figure 1. Maps of the four tropomyosin (Tm) genes and their nonmuscle, nonbrain product(s). Exons are shown as shaded boxes, the 3' untranslated sequence as unshaded boxes and the introns are represented by lines. (A) The fast gene (α -Tmf). Note that 25 exon 1b is unique to Tm5a and Tm5b. (B) Tm5NM gene. (C) The β -TM gene. (D) The TM-4 gene (Taken from Temm-Grove CJ et al 1998 and Percival et al 2000)

Figure 2. Tropomyosin antibody specificity. Tropomyosin antibody specificity in T84 cells and human fibroblasts are shown in Western blots. The specificities of 311 in T84 30 cells (left) and fibroblasts (right) are shown in A and the specificities of α f9d and CG3 antibodies are shown in B and C respectively. The 311 antibody detects Tm6 (40kDa), Tm2 (36kDa) and Tm3 (34kDa) in human fibroblasts. Tm2 is absent in T84 cells. Tm1 (36kDa) was absent from both T84 cells and human fibroblasts. α f9d detects Tm6 35 (40kDa), Tm3 (34kDa), Tm5a (30kDa) and Tm5b (30kDa). CG3 antibody detects 11 possible isoforms that co-migrate at 30kDa.

Figure 3. T84 cell monolayers express a polarised distribution of Tm5a and Tm5b. (A – F) Mature T84 cell monolayers were labelled with α f9d (A and B), 311 (C and D) and CG3 antibodies. The antibody distribution was analysed by Confocal Laser Scanning Microscopy. Images in the vertical plane (xz) are shown on the left and 5 images in the horizontal plane (xy) are shown on the right. The differential staining pattern between α f9d and 311 represents Tm5a and Tm5b. Bar, 10 μ m. (G) The mean apical and central pixel intensity was measured across the apical and central region of the individual monolayers. The apical: central mean pixel intensity ratios for α f9d and 10 311 were compared in co-stained monolayers and are represented as the mean \pm standard deviation for each group. Results represent the average of 8 co-stained monolayers.

Figure 4. Localisation of tropomyosin isoforms in the crypts and villi of the rat duodenum. Sections of rat duodenal tissue were fixed and stained with α f9d (C and D), 15 311, (E and F) and CG3 (G and H) antibodies. Sections through the crypts are on the left and sections through the villi are on the right. A and B represent antibody negative controls. Arrows indicate gastrointestinal epithelial cells. Immunoreactivity is indicated by the blue staining and slides were counterstained with Nuclear fast red. Bar, 10 μ m.

Figure 5. The development of polarisation of tropomyosin isoforms. (A-L) Immunofluorescent confocal microscopy images of T84 cells stained for tropomyosin isoforms at various time points after seeding. All images are in the vertical (xz) plane. At each time point, the images on the left and in the centre are of the same co-stained 25 cells. On the left the 311 antibody (Tm 3, 6) staining is shown. In the centre the α f9d antibody (Tm 3, 5a, 5b, 6) staining is shown. The cells on the right are stained with CG3 antibody (TmNM1-11). (A, B and C) 10 minutes; (D, E and F) 1 hour; (G, H and I) 2 hours; (J, K and L) 24 hours. The arrow indicates a T84 cell in suspension with circumferential staining. Bar, 10 μ m. (M and N) Total protein and specific 30 tropomyosin isoform expression during T84 cell monolayer development. Protein was extracted from T84 cells 1,2,4 and 24 hours and 7 days after seeding. (M) Gel stained with Coomassie blue showing total protein. (N) Western blot immunoblotted with α f9d antibody (Tm 3, 5a, 5b, 6).

35 **Figure 6.** Localisation of tropomyosin isoforms in T84 cells after treatment with jasplakinolide or nocodazole. Immunofluorescent confocal microscopy images of T84

cells stained for tropomyosin isoforms 10 minutes after cell seeding (A-D) and mature T84 cell monolayer (E and F). All images are in the vertical (xz) plane. Cells on the left are stained with 311 antibody (Tm 3,6) and cells on the right are stained with α f9d antibody (Tm 3, 5a, 5b, 6). (A and B) Cells treated with jasplakinolide 1 μ M. (C and D)

- 5 Cells treated with nocodazole 33 μ M. (E and F) T84 cell monolayers treated with 20 μ M cytochalasin D for 3 hours. The arrows indicate a T84 cells in suspension with circumferential staining. Bar, 10 μ m.

Figure 7. T84 cell monolayers co-stained for tropomyosin isoforms and CFTR.

- 10 Immunofluorescent confocal microscopy images of T84 cell monolayers co-stained for tropomyosin isoforms and CFTR. All images are in the vertical plane. (A) α f9d antibody (Tm 3,5a,5b,6). The arrow indicates area of enriched α f9d staining in the apical membrane not associated with CFTR; (B) CFTR antibody. The arrow indicates CFTR located in the cytoplasm; (C) Overlay of image A and image B. Bar, 10 μ m.

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Figure 8. Effect of antisense and nonsense oligonucleotides against Tm5a and Tm5b on the distribution of α f9d antibody staining in T84 cell monolayers. Immunofluorescent confocal microscopy images of T84 cell monolayers. Both images are in the vertical plane. Both monolayers have been stained with α f9d (Tm3, 5a, 5b,

- 20 6). (A) Nonsense oligonucleotide 2 μ M for 24 hours; (B) Antisense oligonucleotide 2 μ M for 24 hours. Bar, 10 μ m. (C and D) Western blot showing the effect of antisense and nonsense oligonucleotides against Tm5a and Tm5b on T84 cells. Protein was extracted from T84 cell monolayers following treatment with either 2 μ M antisense or nonsense oligonucleotides against Tm5a and Tm5b for 24 hours. (C) Gel stained with
25 Coomassie blue showing total protein. (D) Western blot immunoblotted with the α f9d antibody (Tm3, 5a, 5b, 6). (E) Effect of antisense and nonsense oligonucleotides against Tm5a and Tm5b on intensity of apical staining with α f9d antibody in T84 cell monolayers. The apical α f9d antibody staining pixel intensity was determined by confocal microscope in T84 cell monolayers treated with either 2 μ M antisense or
30 nonsense oligonucleotides for 24 hours. The mean \pm 1SD for each group is depicted. (Nonsense 150.86 \pm 48.28, Antisense 53.62 \pm 31.62; p < 0.001)

Figure 9. Effect of antisense and nonsense oligonucleotides against Tm5a and Tm5b on cell surface expression of CFTR and chloride efflux in T84 cell monolayers. (A)

- 35 Enzyme linked CFTR surface expression assays were performed on T84 cell monolayers treated with either 2 μ M antisense or nonsense oligonucleotides for 24

hours. CFTR expression is represented by absorbance at 655nm, normalised to the mean absorbance of the nonsense treated group within individual experiments. The mean \pm 1SD for each group is depicted. (Nonsense 1 \pm 0.42, Antisense 1.49 \pm 0.78; p < 0.001). (B) MQAE chloride efflux assays were performed on control T84 cell monolayers treated with either 2 μ M antisense or nonsense oligonucleotides against Tm5a and Tm5b. Cumulative chloride efflux at 15 minutes is represented by the mean percentage increase in fluorescence from baseline, normalised to the mean percentage increase of the nonsense group, within individual experiments. The mean \pm 1SD for each group is depicted. (Nonsense 1 \pm 0.36, Antisense 1.47 \pm 0.41; p < 0.001)

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Figure 10. Effect of nocodazole treatment on cell surface expression of CFTR and chloride efflux in T84 cell monolayers. Enzyme linked CFTR surface expression assays were performed on forskolin stimulated T84 cell monolayers with and without treatment with 33 μ M nocodazole for 3 hours. CFTR expression is represented by 15 absorbance at 655nm, normalised to the mean absorbance of the control group within individual experiments. The mean \pm SD for each group is depicted. (Control 1.00 \pm 0.29, Nocodazole 0.92 \pm 0.25; p = 0.64). (B) MQAE chloride efflux assays were performed on control T84 cell monolayers and T84 cell monolayers treated with nocodazole 33 μ M for 3 hours. Cumulative chloride efflux at 15 minutes is represented 20 by the mean percentage increase in fluorescence from baseline, normalised to the mean percentage increase of the control group, within individual experiments. The mean \pm SD for each group is depicted. (Control 1.00 \pm 0.22, Nocodazole 1.01 \pm 0.43; p = 0.93).

25 Key to Sequence Listing

- SEQ ID NO:1: *Homo sapiens* tropomyosin 1 (alpha) (TPM 1) gene sequence;
SEQ ID NO:2: *Homo sapiens* tropomyosin 2 (beta) (TPM 2) gene sequence;
SEQ ID NO:3: *Homo sapiens* tropomyosin 3 (TPM 3) gene sequence;
30 SEQ ID NO:4: *Homo sapiens* tropomyosin 4 (TPM 4) gene sequence.

Detailed Description of the Preferred Embodiments

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Tropomyosins

- 15 The present invention is based on the finding that insertion, retention or maintenance of proteins in the surface membrane of a cell is regulated by tropomyosin. This finding provides the basis for diagnostic and therapeutic methods relating to diseases that are caused by abnormal insertion or functioning of cell surface proteins.
- 20 Tropomyosins (TMs) are a diverse group of proteins found in all eukaryotic cells, with distinct isoforms found in muscle (skeletal, cardiac and smooth), brain and various non-muscle cells. They are elongated proteins that possess a simple dimeric α -helical coiled coil structure along their entire length. The coiled coil structure is based on a repeated pattern of seven amino acids with hydrophobic residues at the first and fourth
- 25 positions and is highly conserved in all TM isoforms found in eukaryotic organisms from yeast to man with a prominent seven-residue periodicity (five motifs). Different isoforms are produced by differential splicing; e.g isoforms of α -tropomyosin differ in striated and smooth muscle.
- 30 TMs are associated with the thin filaments in the sarcomeres of muscle cells and the microfilaments of muscle cells. The TMs bind to themselves in a head-to-tail manner, and lie in the grooves of F-actin, with each molecule interacting with six or seven actin monomers.
- 35 The function of TM in skeletal and cardiac muscle is, in association with the troponin complex (troponins T, C and I), to regulate the calcium-sensitive interaction of actin

and myosin. Under resting intracellular calcium ion concentrations, the troponin-tropomyosin complex inhibits actomyosin ATPase activity. When a stimulus induces calcium ion release in the muscle cell, troponin-C binds additional calcium ions and a conformational change is transmitted through the troponin-tropomyosin complex which

- 5 releases the inhibition of actomyosin ATPase activity, resulting in contraction.

In contrast to the skeletal and cardiac muscle, the biological functions of smooth muscle and non-muscle TMs are poorly understood. Smooth muscle and non-muscle cells are devoid of a troponin complex and the phosphorylation of the light chain of

- 10 myosins appears to be the major calcium-sensitive regulatory mechanism controlling the interaction of actin and myosin. These differences in the regulation of contractile apparatus of various cell types appear to require structurally as well as functionally distinct forms of TM.

- 15 When used herein the term "tropomyosin" is intended to encompass all isoforms of the protein. For example, the term encompasses all isoforms encoded by the mammalian genes TPM 1 (also known as the alpha-TM gene) (MacLeod and Gooding, 1988, Mol. Cell. Biol. 8, 433-440), TPM 2 (also known as the beta-TM gene) (MacLeod *et al.*, 1985, Proc. Natl. Acad. Sci. USA 82, 7835-7839), TPM 3 (also known as the gamma-
- 20 TM gene) (Clayton *et al.*, 1988, J. Mol. Biol. 201, 507-515), and TPM 4 (also known as the delta-TM gene) (MacLeod *et al.*, 1987, J. Mol. Biol. 194, 1-10).

There are at least 40 tropomyosin isoforms that are derived from these four genes by alternative splicing (Figure 1). See, for example, Lees-Miller and Helfman, 1991,

- 25 Bioessays 13(9):429-437. Although tropomyosin isoforms have a high degree of similarity, there are some differences in the actin binding and head-tail binding domains. The various tropomyosin isoforms have different binding affinities for actin and this is thought to result in a differential effect on the stability of actin microfilaments. In addition, the tropomyosin position on the actin microfilament may 30 modulate actin's role in cell motility and cytoskeletal remodelling. Once inserted, tropomyosins influence the interaction between actin and other actin binding proteins. For example, high molecular weight tropomyosins are protective against the severing activity of the actin binding protein gelsolin.
- 35 The sequences of the human TPM1, TMP2, TPM3 and TPM4 genes are shown in SEQ ID NOS 1 to 4 respectively. These sequences are representative examples only and are

not intended to limit the scope of the present invention. The methods of the present invention may be targeted to other human or non-human tropomyosin sequences.

Diagnostic analysis

5

In one aspect the present invention relates to a method for predicting the likelihood that an individual has a predisposition to a disease caused by abnormal insertion, retention or function of a cell surface protein, or for aiding in the diagnosis of such a disease.

- 10 In one embodiment the diagnostic method comprises the steps of obtaining a polynucleotide sample from an individual to be assessed and analysing a tropomyosin gene.

The genetic material to be assessed can be obtained from any nucleated cell from the
15 individual. For assay of genomic DNA, virtually any biological sample (other than pure red blood cells) is suitable. For example, convenient tissue samples include whole blood, semen, saliva, tears, urine, fecal material, sweat, skin, testis, placenta, kidney and hair. For assay of cDNA or mRNA, the tissue sample is preferably obtained from an organ in which the target nucleic acid is expressed. For example, epithelial cells are
20 suitable sources for obtaining cDNA for tropomyosin genes.

The analysis of the tropomyosin gene may require amplification of DNA from target samples. This can be accomplished by e.g., PCR. See generally PCR Technology: Principles and Applications for DNA Amplification (ed. H. A. Erlich, Freeman Press,
25 New York, N.Y., 1992); PCR Protocols: A Guide to Methods and Applications (eds. Innis, et al., Academic Press, San Diego, Calif., 1990); Mattila *et al.*, Nucleic Acids Res. 19, 4967 (1991); Eckert *et al.*, PCR Methods and Applications 1, 17 (1991); PCR (eds. McPherson *et al.*, IRL Press, Oxford); and U.S. Pat. No. 4,683,202.

- 30 Other suitable amplification methods include the ligase chain reaction (LCR) (see Wu and Wallace, Genomics 4, 560 (1989), Landegren *et al.*, Science 241, 1077 (1988), transcription amplification (Kwoh *et al.*, Proc. Natl. Acad. Sci. USA 86, 1173 (1989)), and self-sustained sequence replication (Guatelli *et al.*, Proc. Nat. Acad. Sci. USA, 87, 1874 (1990)) and nucleic acid based sequence amplification (NASBA). The latter two
35 amplification methods involve isothermal reactions based on isothermal transcription,

which produce both single stranded RNA (ssRNA) and double stranded DNA (dsDNA) as the amplification products in a ratio of about 30 or 100 to 1, respectively.

The nucleotide which occupies a polymorphic site of interest can be identified by a
5 variety methods, such as Southern analysis of genomic DNA; direct mutation analysis by restriction enzyme digestion; Northern analysis of RNA; denaturing high pressure liquid chromatography (DHPLC); gene isolation and sequencing; hybridization of an allele-specific oligonucleotide with amplified gene products; exon trapping, single base extension (SBE); or analysis of the tropomyosin protein.

10 In another embodiment the diagnostic method involves analysing the polarised distribution of tropomyosin in the cells of the individual. This analysis may be conducted, for example, by antibody staining of a particular tropomyosin isoform within cells (preferably epithelial cells) derived from the individual being tested.

15 Tropomyosin antagonists/agonists
The therapeutic methods of the present invention involve the use of tropomyosin modulators (e.g. agonists or antagonists).

20 In certain embodiments, combinatorial libraries of potential modulators will be screened for an ability to bind to a tropomyosin or to modulate activity. Conventionally, new chemical entities with useful properties are generated by identifying a chemical compound (called a "lead compound") with some desirable
25 property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

In one preferred embodiment, high throughput screening methods involve providing a
30 library containing a large number of potential therapeutic compounds (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more assays to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual
35 therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library, such as a polypeptide (e.g., mutein) library, is formed by combining a

- 5 set of chemical building blocks called amino acids in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks (Gallop *et al.*, 1994, *J. Med. Chem.* 37(9):1233-1251).

10

- Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries, peptoids, encoded peptides, random bio-oligomers, nonpeptidal peptidomimetics, analogous organic syntheses of small compound libraries, nucleic acid libraries, peptide nucleic acid libraries, antibody libraries, carbohydrate libraries and small organic molecule libraries.

- The assays to identify modulators are amenable to high throughput screening. Preferred assays thus detect enhancement or inhibition of tropomyosin gene
20 transcription, inhibition or enhancement of polypeptide expression, and inhibition or enhancement of polypeptide activity.

- High throughput assays for the presence, absence, quantification, or other properties of particular nucleic acids or protein products are well known to those of skill in the art.
25 Similarly, binding assays and reporter gene assays are similarly well known. Thus, e.g., U.S. Patent No. 5,559,410 discloses high throughput screening methods for proteins, U.S. Patent No. 5,585,639 discloses high throughput screening methods for nucleic acid binding (i.e., in arrays), while U.S. Patent Nos. 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

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- In addition, high throughput screening systems are commercially available (see, e.g., Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures, including all sample and reagent
35 pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detectors) appropriate for the assay. These configurable systems provide high

throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols for various high throughput systems. Thus, e.g., Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding,
5 and the like.

Protein or Peptide inhibitors

In one embodiment, modulators are proteins, often naturally occurring proteins or
10 fragments of naturally occurring proteins. Thus, e.g., cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of proteins may be made for screening in the methods of the invention. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins
15 being especially preferred. Particularly useful test compound will be directed to the class of proteins to which the target belongs, e.g., substrates for enzymes or ligands and receptors.

In a preferred embodiment, modulators are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since
25 generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous
30 agents.

In one embodiment, peptidyl tropomyosin inhibitors are chemically or recombinantly synthesized as oligopeptides (approximately 10-25 amino acids in length) derived from the tropomyosin sequence. Alternatively, tropomyosin fragments are produced by
35 digestion of native or recombinantly produced tropomyosin by, for example, using a protease, e.g., trypsin, thermolysin, chymotrypsin, or pepsin. Computer analysis (using

commercially available software, e.g. MacVector, Omega, PCGene, Molecular Simulation, Inc.) is used to identify proteolytic cleavage sites. The proteolytic or synthetic fragments can comprise as many amino acid residues as are necessary to partially or completely inhibit tropomyosin function. Preferred fragments will

- 5 comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length.

Protein or peptide inhibitors may also be dominant-negative mutants of tropomyosin. The term "dominant-negative mutant" refers to a tropomyosin polypeptide that has
10 been mutated from its natural state and that interacts with a protein that tropomyosin normally interacts with thereby preventing endogenous native tropomyosin from forming the interaction.

Anti-tropomyosin Antibodies

15

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')₂, and Fv which are capable of binding an epitopic determinant of tropomyosin. These antibody fragments retain some ability to selectively bind with its antigen and are defined as follows:

20

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

25 (2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

30 (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab)2 is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains;
35 and

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

- 5 Methods of making these fragments are known in the art. (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference).

10 Antibodies of the present invention can be prepared using intact tropomyosin or fragments thereof as the immunizing antigen. A peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis and is purified and conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide 15 may then be used to immunize the animal (e.g., a mouse or a rabbit).

If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the 20 immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, *et al.*, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, 1991, incorporated by reference).

Monoclonal antibodies may be prepared using any technique which provides for the 25 production of antibody molecules by continuous cell lines in culture, such as, for example, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler *et al.* *Nature* 256, 495-497, 1975; Kozbor *et al.*, *J. Immunol. Methods* 81, 31-42, 1985; Cote *et al.*, *Proc. Natl. Acad. Sci. USA* 80, 2026-2030, 1983; Cole *et al.*, *Mol. Cell Biol.* 62, 109-120, 1984).

30 Methods known in the art allow antibodies exhibiting binding for tropomyosin to be identified and isolated from antibody expression libraries. For example, a method for the identification and isolation of an antibody binding domain which exhibits binding to tropomyosin is the bacteriophage lambda vector system. This vector system has 35 been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in *Escherichia coli* (Huse, *et al.*, *Science*, 246:1275-1281, 1989) and from

the human antibody repertoire (Mullinax, *et al.*, Proc. Nat. Acad. Sci., 87:8095-8099, 1990). This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using 5 techniques well understood by those having ordinary skill in the art and will not be repeated here. Details of these techniques are described in such references as Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis, Edited by Roger H. Kennett, *et al.*, Plenum Press, 1980; and U.S. 4,172,124, incorporated by reference.

10 In addition, methods of producing chimeric antibody molecules with various combinations of "humanized" antibodies are known in the art and include combining murine variable regions with human constant regions (Cabilly, *et al.* Proc. Natl. Acad. Sci. USA, 81:3273, 1984), or by grafting the murine-antibody complementarity 15 determining regions (CDRs) onto the human framework (Riechmann, *et al.*, Nature 332:323, 1988).

Antisense compounds

20 The term "antisense compounds" encompasses DNA or RNA molecules that are complementary to at least a portion of a tropomyosin mRNA molecule (Izant and Weintraub, Cell 36:1007-15, 1984; Izant and Weintraub, Science 229(4711):345-52, 1985) and capable of interfering with a post-transcriptional event such as mRNA translation. Antisense oligomers complementary to at least about 15 contiguous 25 nucleotides of tropomyosin-encoding mRNA are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target tropomyosin-producing cell. The use of antisense methods is well known in the art (Marcus-Sakura, Anal. Biochem. 172: 289, 1988). Preferred antisense nucleic acid will comprise a nucleotide sequence that is complementary to at 30 least 15 contiguous nucleotides of a sequence encoding the amino acid sequence set forth in SEQ ID NO:1 to SEQ ID NO:4.

Catalytic nucleic acids

35 The term catalytic nucleic acid refers to a DNA molecule or DNA-containing molecule (also known in the art as a "DNAzyme") or an RNA or RNA-containing molecule (also

known as a "ribozyme") which specifically recognizes a distinct substrate and catalyzes the chemical modification of this substrate. The nucleic acid bases in the catalytic nucleic acid can be bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art.

5

Typically, the catalytic nucleic acid contains an antisense sequence for specific recognition of a target nucleic acid, and a nucleic acid cleaving enzymatic activity (also referred to herein as the "catalytic domain"). To achieve specificity, preferred ribozymes and DNAzymes will comprise a nucleotide sequence that is complementary 10 to at least about 12-15 contiguous nucleotides of a sequence encoding a tropomyosin isoform.

The types of ribozymes that are particularly useful in this invention are the hammerhead ribozyme (Haseloff and Gerlach 1988, Perriman *et al.*, 1992) and the 15 hairpin ribozyme (Shippy *et al.*, 1999).

The ribozymes of this invention and DNA encoding the ribozymes can be chemically synthesized using methods well known in the art. The ribozymes can also be prepared from a DNA molecule (that upon transcription, yields an RNA molecule) operably 20 linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention is a nucleic acid molecule, i.e., DNA or cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with RNA 25 polymerase and nucleotides. In a separate embodiment, the DNA can be inserted into an expression cassette or transcription cassette. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase. Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also 30 renders the ribozyme resistant to endonuclease activity.

RNA inhibitors

dsRNA is particularly useful for specifically inhibiting the production of a particular 35 protein. Although not wishing to be limited by theory, Dougherty and Parks (1995) have provided a model for the mechanism by which dsRNA can be used to reduce

protein production. This model has recently been modified and expanded by Waterhouse *et al.* (1998). This technology relies on the presence of dsRNA molecules that contain a sequence that is essentially identical to the mRNA of the gene of interest, in this case an mRNA encoding a tropomyosin protein. Conveniently, the dsRNA can 5 be produced in a single open reading frame in a recombinant vector or host cell, where the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a loop structure. The design and production of suitable dsRNA molecules targeted against tropomyosin is well within the capacity of a person 10 skilled in the art, particularly considering Dougherty and Parks (1995), Waterhouse *et al.* (1998), WO 99/32619, WO 99/53050, WO 99/49029, and WO 01/34815.

As used herein, the terms "small interfering RNA", and "RNAi" refer to homologous double stranded RNA (dsRNA) that specifically targets a gene product, thereby 15 resulting in a null or hypomorphic phenotype. Specifically, the dsRNA comprises two short nucleotide sequences derived from the target RNA encoding tropomyosin and having self-complementarity such that they can anneal, and interfere with expression of a target gene, presumably at the post-transcriptional level. RNAi molecules are described by Fire *et al.*, Nature 391, 806-811, 1998, and reviewed by Sharp, Genes & 20 Development, 13, 139-141, 1999).

Small molecule inhibitors

Candidate compounds may be organic molecules, preferably small organic compounds 25 having a molecular weight of more than 100 and less than about 2,500 daltons. Preferred small molecules are less than 2000, or less than 1500 or less than 1000 or less than 500 Daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two 30 of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

In one embodiment, the present invention involves screening small molecule chemodiversity represented within libraries of parent and fractionated natural product extracts, to detect bioactive compounds as potential candidates for further characterization.

5

In one embodiment of the present invention, the candidate compound is obtained from expression products of a gene library, a low molecular weight compound library (such as the low molecular weight compound library of ChemBridge Research Laboratories), a cell extract, microorganism culture supernatant, bacterial cell components and the like. In one particular embodiment, the candidate compound is obtained from an extract of a strain of Enteropathogenic *E. coli* (EPEC).

Methods of screening for tropomyosin agonists/antagonists

15 *Screening protocol based on polarised distribution of tropomyosin*

An example of a screening method in which the ability of a candidate compound to inhibit tropomyosin function may involve an analysis of the effect of the compound on polarised distribution of tropomyosin within a cell.

20

For example, cells expressing a labelled tropomyosin isoform of interest may be exposed to candidate compounds and monitored for the loss of polarised distribution of that tropomyosin isoform. The labelled tropomyosin isoform may be generated, for example, by expression of a fusion construct comprising tropomyosin linked to a fluorescent compound (such as the green fluorescent protein (GFP)) within the cell. Those skilled in the art would understand that other detectable labels may be used in this screening assay.

30 Alternatively, a sample of cells may be exposed to a candidate compound, and the distribution of the tropomyosin isoform of interest determined by antibody staining.

Screening protocol based on expression of tropomyosin

An example of a screening method in which the ability of a candidate compound to 35 inhibit tropomyosin expression may involve the following steps:

- (i) contacting a candidate compound with cells capable of expressing tropomyosin,
- (ii) measuring the amount of expression of tropomyosin in the cells brought into contact with the candidate compound and comparing this amount of expression with
5 the amount of expression (control amount of expression) of tropomyosin in the corresponding control cells not brought into contact with an investigational substance, and
- (iii) selecting a candidate compound showing a reduced amount of expression of
10 tropomyosin as compared with the amount of control expression on the basis of the result of the above step (ii).

The cells used in this screening method may be any cells that can express tropomyosin, irrespective of the difference between natural and recombinant genes. Moreover, the
15 derivation of the tropomyosin is not particularly limited. The cells may be human derived, or may derive from mammals other than humans such as mice, or from other organisms. Examples of suitable human cells are hematopoietic cells including mast cells. Moreover, transformed cells that contain expression vectors comprising nucleic acid sequences that encode tropomyosin may also be used.

20 The conditions for allowing the candidate compound to come into contact with the cells that can express tropomyosin are not limited, but it is preferable to select from among culture conditions (temperature, pH, culture composition, etc.) which will not kill the applicable cells, and in which the tropomyosin genes can be expressed.

25 The term "reduced" refers not only the comparison with the control amount of expression, but also encompasses cases where no tropomyosin is expressed at all. Specifically, this includes circumstances wherein the amount of expression of tropomyosin is substantially zero.

30 The amount of expression of tropomyosin can be assessed either by measuring the amount of expression of a tropomyosin gene (mRNA) or by measuring the amount of a tropomyosin protein produced. In addition, the method to measure the amount of tropomyosin need not be a method to directly measure the amount of expression of
35 gene (mRNA) or the amount of protein produced, but may be any method that reflects these.

- Specifically, to measure the amount of expression of tropomyosin (detection and assay), the amount of expression of tropomyosin mRNA may be measured utilizing DNA array or well-known methods such as the Northern blot method, as well as the
- 5 RT-PCR method that utilizes oligonucleotides having nucleotide sequences complementary to the nucleotide sequence of the applicable tropomyosin mRNA. Moreover, the amount of tropomyosin protein may be measured by implementing such well-known methods as the Western blot method utilizing an anti-tropomyosin antibody.
- 10 The measurement of the amount of expression of tropomyosin (detection and assay) may be implemented by measuring the activity of proteins derived from marker genes, using a cell line into which have been introduced fused genes comprising the marker genes such as reporter genes (e.g., luciferase genes, chloramphenicol-acetyltransferase
- 15 genes, β -glucuronidase genes, β -galactosidase genes and aequorin genes) linked to the suppression region of the tropomyosin gene.
- Screening protocol based on binding of tropomyosin to one or more of its binding partners*
- 20 In one embodiment, tropomyosin agonists or antagonists are identified by screening for candidate compounds which interfere with the binding of tropomyosin to a tropomyosin binding partner. An example of a suitable tropomyosin binding partner is actin.
- 25 Standard solid-phase ELISA assay formats are particularly useful for identifying antagonists of the protein-protein interaction. In accordance with this embodiment, one of the binding partners, e.g. an actin filament, is immobilized on a solid matrix, such as, for example an array of polymeric pins or a glass support. Conveniently, the
- 30 immobilized binding partner is a fusion polypeptide comprising Glutathione-S-transferase (GST; e.g. a CAP-ERK fusion), wherein the GST moiety facilitates immobilization of the protein to the solid phase support. The second binding partner (e.g. tropomyosin) in solution is brought into physical relation with the immobilized protein to form a protein complex, which complex is detected using antibodies directed
- 35 against the second binding partner. The antibodies are generally labelled with fluorescent molecules or conjugated to an enzyme (e.g. horseradish peroxidase), or

- alternatively, a second labelled antibody can be used that binds to the first antibody. Conveniently, the second binding partner is expressed as a fusion polypeptide with a FLAG or oligo-histidine peptide tag, or other suitable immunogenic peptide, wherein antibodies against the peptide tag are used to detect the binding partner. Alternatively,
5 oligo-HIS tagged protein complexes can be detected by their binding to nickel-NTA resin (Qiagen), or FLAG-labeled protein complexes detected by their binding to FLAG M2 Affinity Gel (Kodak). It will be apparent to the skilled person that the assay format described herein is amenable to high throughput screening of samples, such as, for example, using a microarray of bound peptides or fusion proteins.
- 10 A two-hybrid assay as described in US Patent No. 6,316,223 may also be used to identify compounds that interfere with the binding of tropomyosin to one of its binding partners. The basic mechanism of this system is similar to the yeast two hybrid system. In the two-hybrid system, the binding partners are expressed as two distinct fusion
15 proteins in a mammalian host cell. In adapting the standard two-hybrid screen to the present purpose, a first fusion protein consists of a DNA binding domain which is fused to one of the binding partners, and a second fusion protein consists of a transcriptional activation domain fused to the other binding partner. The DNA binding domain binds to an operator sequence which controls expression of one or more reporter genes. The
20 transcriptional activation domain is recruited to the promoter through the functional interaction between binding partners. Subsequently, the transcriptional activation domain interacts with the basal transcription machinery of the cell, thereby activating expression of the reporter gene(s), the expression of which can be determined. Candidate bioactive agents that modulate the protein-protein interaction between the
25 binding partners are identified by their ability to modulate transcription of the reporter gene(s) when incubated with the host cell. Antagonists will prevent or reduce reporter gene expression, while agonists will enhance reporter gene expression. In the case of small molecule modulators, these are added directly to the cell medium and reporter gene expression determined. On the other hand, peptide modulators are expressible
30 from nucleic acid that is transfected into the host cell and reporter gene expression determined. In fact, whole peptide libraries can be screened in transfected cells.

Alternatively, reverse two hybrid screens, such as, for example, described by Vidal *et al.*, Proc. Natl Acad. Sci USA 93, 10315-10320, 1996, may be employed to identify
35 antagonist molecules. Reverse hybrid screens differ from froward screens supra in so far as they employ a counter-selectable reporter gene, such as for example, CYH2 or

- LYS2, to select against the protein-protein interaction. Cell survival or growth is reduced or prevented in the presence of a non-toxic substrate of the counter-selectable reporter gene product, which is converted by said gene product to a toxic compound. Accordingly, cells in which the protein-protein interaction of the invention does not
- 5 occur, such as in the presence of an antagonist of said interaction, survive in the presence of the substrate, because it will not be converted to the toxic product. For example, a portion/fragment of tropomyosin that binds to actin is expressed as a DNA binding domain fusion, such as with the DNA binding domain of GAL4; and the portion of actin that binds tropomyosin is expressed as an appropriate transcription
- 10 activation domain fusion polypeptide (e.g. with the GAL4 transcription activation domain). The fusion polypeptides are expressed in yeast in operable connection with the URA3 counter-selectable reporter gene, wherein expression of URA3 requires a physical relation between the GAL4 DNA binding domain and transcriptional activation domain. This physical relation is achieved, for example, by placing reporter
- 15 gene expression under the control of a promoter comprising nucleotide sequences to which GAL4 binds. Cells in which the reporter gene is expressed do not grow in the presence of uracil and 5-fluororotic acid (5-FOA), because the 5-FOA is converted to a toxic compound. Candidate peptide inhibitor(s) are expressed in libraries of such cells, wherein cells that grow in the presence of uracil and 5-FOA are retained for further
- 20 analysis, such as, for example, analysis of the nucleic acid encoding the candidate peptide inhibitor(s). Small molecules that antagonize the interaction are determined by incubating the cells in the presence of the small molecules and selecting cells that grow or survive of cells in the presence of uracil and 5-FOA.
- 25 Alternatively, a protein recruitment system, such as that described in U.S. Patent No. 5, 776, 689 to Karin *et al.*, may be used. In a standard protein recruitment system, a protein-protein interaction is detected in a cell by the recruitment of an effector protein, which is not a transcription factor, to a specific cell compartment. Upon translocation of the effector protein to the cell compartment, the effector protein activates a reporter
- 30 molecule present in that compartment, wherein activation of the reporter molecule is detectable, for example, by cell viability, indicating the presence of a protein-protein interaction.

More specifically, the components of a protein recruitment system include a first

35 expressible nucleic acid encoding a first fusion protein comprising the effector protein and one of the binding partners (e.g. actin or a portion thereof), and a second

- expressible nucleic acid molecule encoding a second fusion protein comprising a cell compartment localization domain and the other binding partner (e.g. tropomyosin or a portion thereof). A cell line or cell strain in which the activity of an endogenous effector protein is defective or absent (e.g. a yeast cell or other non-mammalian cell), is 5 also required, so that, in the absence of the protein-protein interaction, the reporter molecule is not expressed.

- A complex is formed between the fusion polypeptides as a consequence of the interaction between the binding partners, thereby directing translocation of the complex 10 to the appropriate cell compartment mediated by the cell compartment localization domain (e.g. plasma membrane localization domain, nuclear localization domain, mitochondrial membrane localization domain, and the like), where the effector protein then activates the reporter molecule. Such a protein recruitment system can be practiced in essentially any type of cell, including, for example, mammalian, avian, insect and 15 bacterial cells, and using various effector protein/reporter molecule systems.

- For example, a yeast cell based assay is performed, in which the interaction between tropomyosin and one or more of its binding partners results in the recruitment of a guanine nucleotide exchange factor (GEF or C3G) to the plasma membrane, wherein 20 GEF or C3G activates a reporter molecule, such as Ras, thereby resulting in the survival of cells that otherwise would not survive under the particular cell culture conditions. Suitable cells for this purpose include, for example, *Saccharomyces cerevisiae cdc25-2* cells, which grow at 36°C only when a functional GEF is expressed therein, Petitjean *et al.*, *Genetics* 124, 797-806, 1990). Translocation of the GEF to the 25 plasma membrane is facilitated by a plasma membrane localization domain. Activation of Ras is detected, for example, by measuring cyclic AMP levels in the cells using commercially available assay kits and/or reagents. To detect antagonists of the protein-protein interaction of the present invention, duplicate incubations are carried out in the presence and absence of a test compound, or in the presence or absence of expression 30 of a candidate antagonist peptide in the cell. Reduced survival or growth of cells in the presence of a candidate compound or candidate peptide indicates that the peptide or compound is an antagonist of the interaction between tropomyosin and one or more of its binding partners.
- 35 A "reverse" protein recruitment system is also contemplated, wherein modified survival or modified growth of the cells is contingent on the disruption of the protein-protein

- interaction by the candidate compound or candidate peptide. For example, NIH 3T3 cells that constitutively express activated Ras in the presence of GEF can be used, wherein the absence of cell transformation is indicative of disruption of the protein complex by a candidate compound or peptide. In contrast, NIH 3T3 cells that
- 5 constitutively express activated Ras in the presence of GEF have a transformed phenotype (Aronheim *et al.*, Cell, 78, 949-961, 1994)

In yet another embodiment, small molecules are tested for their ability to interfere with binding of tropomyosin to one of its binding partners, by an adaptation of plate agar diffusion assay described by Vidal and Endoh, TIBS 17, 374-381, 1999, which is incorporated herein by reference.

Screening method based on myosin ATPase activity

- 15 In an adaptation of the screening protocol based on binding of tropomyosin to one or more of its binding partners, the method involves the addition of myosin to the reaction mix and detection of myosin ATPase activity.

For example, a tropomyosin isoform may be incubated with actin filaments and specific
20 myosins. Myosin ATPase activity is then measured in the presence and absence of the candidate compounds. Under normal conditions, tropomyosin inhibits myosin ATPase activity. Accordingly, compounds that interact with tropomyosin and prevent this inhibitory activity will result in increased myosin ATPase activity. Such compounds may be selected for further screening and/or characterisation. Suitable positive control
25 reactions may be performed without tropomyosin or with an inappropriate tropomyosin isoform to eliminate anti-myosin effects.

Therapeutic methods

- 30 The tropomyosin agonists or antagonists identified by the methods of the present invention can be used therapeutically for diseases caused by abnormal insertion, retention or function of cell surface proteins. The term "therapeutically" or as used herein in conjunction with the tropomyosin agonists or antagonists of the invention denotes both prophylactic as well as therapeutic administration. Thus, tropomyosin
35 agonists/antagonists can be administered to high-risk patients in order to lessen the

likelihood and/or severity of a disease or administered to patients already evidencing active disease.

- Diseases or conditions of humans or other species which can be treated with agonists
5 or antagonists of tropomyosin function include, but are not limited to: cystic fibrosis, multiple sclerosis, polycistic kidney disease, viral infection, bacterial infection, reperfusion injury, Menkes Disease, Wilson's Disease, diabetes, myotonic dystrophies, epilepsy or a mood disorders such as depression, bipolar disorder or dysthymic disorder.

10

Modes of Administration

In the case where the candidate compound is in the form of a low molecular weight compound, a peptide or a protein such as an antibody, the substance can be formulated
15 into the ordinary pharmaceutical compositions (pharmaceutical preparations) which are generally used for such forms, and such compositions can be administered orally or parenterally. Generally speaking, the following dosage forms and methods of administration can be utilized

- 20 The dosage form includes such representative forms as solid preparations, e.g. tablets, pills, powders, fine powders, granules, and capsules, and liquid preparations, e.g. solutions, suspensions, emulsions, syrups, and elixirs. These forms can be classified by the route of administration into said oral dosage forms or various parenteral dosage forms such as transnasal preparations, transdermal preparations, rectal preparations
25 (suppositories), sublingual preparations, vaginal preparations, injections (intravenous, intraarterial, intramuscular, subcutaneous, intradermal) and drip injections. The oral preparations., for instance, may for example be tablets, pills, powders, fine powders, granules, capsules, solutions, suspensions, emulsions, syrups, etc. and the rectal and vaginal preparations include tablets, pills, and capsules, among others. The transdermal
30 preparations may not only be liquid preparations, such as lotions, but also be semi-solid preparations, such as creams, ointments, and so forth.

The injections may be made available in such forms as solutions, suspensions and emulsions, and as vehicles, sterilized water, water-propylene glycol, buffer solutions,
35 and saline of 0.4 weight % concentration can be mentioned as examples. These injections, in such liquid forms, may be frozen or lyophilized. The latter products,

obtained by lyophilization, are extemporaneously reconstituted with distilled water for injection or the like and administered. The above forms of pharmaceutical composition (pharmaceutical preparation) can be prepared by formulating the compound having tropomyosin inhibitory action and a pharmaceutically acceptable carrier in the manner

5 established in the art. The pharmaceutically acceptable carrier includes various excipients, diluents, fillers, extenders, binders, disintegrators, wetting agents, lubricants, and dispersants, among others. Other additives which are commonly used in the art can also be formulated. Depending on the form of pharmaceutical composition to be produced, such additives can be judiciously selected from among various

10 stabilizers, fungicides, buffers, thickeners, pH control agents, emulsifiers, suspending agents, antiseptics, flavors, colors, tonicity control or isotonizing agents, chelating agents and surfactants, among others.

- The pharmaceutical composition in any of such forms can be administered by a route
- 15 suited to the objective disease, target organ, and other factors. For example, it may be administered intravenously, intraarterially, subcutaneously, intradermally, intramuscularly or via airways. It may also be directly administered topically into the affected tissue or even orally or rectally.
- 20 The dosage and dosing schedule of such a pharmaceutical preparation vary with the dosage form, the disease or its symptoms, and the patient's age and body weight, among other factors, and cannot be stated in general terms. The usual dosage, in terms of the daily amount of the active ingredient for an adult human, may range from about 0.0001 mg to about 500 mg, preferably about 0.001 mg to about 100 mg, and this
- 25 amount can be administered once a day or in a few divided doses daily.

When the substance having tropomyosin inhibitory activity is in the form of a polynucleotide such as an antisense compound, the composition may be provided in the form of a drug for gene therapy or a prophylactic drug. Recent years have witnessed a

30 number of reports on the use of various genes, and gene therapy is by now an established technique.

The drug for gene therapy can be prepared by introducing the object polynucleotide into a vector or transfecting appropriate cells with the vector. The modality of

35 administration to a patient is roughly divided into two modes, viz. The mode applicable to (1) the case in which a non-viral vector is used and the mode applicable to

(2) the case in which a viral vector is used. Regarding the case in which a viral vector is used as said vector and the case in which a non-viral vector is used, respectively, both the method of preparing a drug for gene therapy and the method of administration are dealt with in detail in several books relating to experimental protocols [e.g.

- 5 "Bessatsu Jikken Igaku, Idenshi Chiryo-no-Kosogijutsu (Supplement to Experimental Medicine, Fundamental Techniques of Gene Therapy), Yodosha, 1996; Bessatsu Jikken Igaku: Idenshi Donyu & Hatsugen Kaiseki Jikken-ho (Supplement to Experimental Medicine: Experimental Protocols for Gene Transfer & Expression Analysis), Yodosha, 1997; Japanese Society for Gene Therapy (ed.): Idenshi Chiryo Kaihatsu
- 10 Kenkyn Handbook (Research Handbook for Development of Gene Therapies), NTS, 1999, etc.].

When using a non-viral vector, any expression vector capable of expressing the anti-tropomyosin nucleic acid may be used. Suitable examples include pCAGGS [Gene 15 108, 193-200 (1991)], pBK-CMV, pcDNA 3.1, and pZeoSV (Invitrogen, Stratagene).

Transfer of a polynucleotide into the patient can be achieved by inserting the object polynucleotide into such a non-viral vector (expression vector) in the routine manner and administering the resulting recombinant expression vector. By so doing, the object 20 polynucleotide can be introduced into the patient's cells or tissue.

More particularly, the method of introducing the polynucleotide into cells includes the calcium phosphate transfection (coprecipitation) technique and the DNA (polynucleotide) direct injection method using a glass microtube, among others.

25 The method of introducing a polynucleotide into a tissue includes the polynucleotide transfer technique using internal type liposomes or electrostatic type liposomes, the HVJ-liposome technique, the modified HVJ-liposome (HVJ-AVE liposome) technique, the receptor-mediated polynucleotide transfer technique, the technique which 30 comprises transferring the polynucleotide along with a vehicle (metal particles) into cells with a particle gun, the naked-DNA direct transfer technique, and the transfer technique using a positively charged polymer, among others.

Suitable viral vectors include vectors derived from recombinant adenoviruses and 35 retrovirus. Examples include vectors derived from DNA or RNA viruses such as detoxicated retrovirus, adenovirus, adeno-associated virus, herpesvirus, vaccinia virus,

poxvirus, poliovirus, sindbis virus, Sendai virus, SV40, human immunodeficiency virus (HIV) and so forth. The adenovirus vector, in particular, is known to be by far higher in infection efficiency than other viral vectors and, from this point of view, the adenovirus vector is preferably used.

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Transfer of the polynucleotide into the patient can be achieved by introducing the object polynucleotide into such a viral vector and infecting the desired cells with the recombinant virus obtained. In this manner, the object polynucleotide can be introduced into the cells.

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The method of administering the thus-prepared drug for gene therapy to the patient includes the *in vivo* technique for introducing the drug for gene therapy directly into the body and the *ex vivo* technique which comprises withdrawing certain cells from a human body, introducing the drug for gene therapy into the cells *in vitro* and returning the cells into the human body [Nikkei Science, April, 1994 issue, 20-45; Pharmaceuticals Monthly, 36(1), 23-48, 1994; Supplement to Experimental Medicine, 12(15), 1994; Japanese Society for Gene Therapy (ed.): Research Handbook for Development of Gene Therapies, NTS, 19991]. For use in the prevention or treatment of an inflammatory disease to which the present invention is addressed, the drug is preferably introduced into the body by the *in vivo* technique.

When the *in vivo* method is used, the drug can be administered by a route suited to the object disease, target organ or the like. For example, it can be administered intravenously, intraarterially, subcutaneously or intramuscularly, for instance, or may be directly administered topically into the affected tissue.

The drug for gene therapy can be provided in a variety of pharmaceutical forms according to said routes of administration. In the case of an injectable form, for instance, an injection can be prepared by the per se established procedure, for example by dissolving the active ingredient polynucleotide in a solvent, such as a buffer solution, e.g. PBS, physiological saline, or sterile water, followed by sterilizing through a filter where necessary, and filling the solution into sterile vials. Where necessary, this injection may be supplemented with the ordinary carrier or the like. In the case of liposomes such as HVJ-liposome, the drug can be provided in various liposome-entrapped preparations in such forms as suspensions, frozen preparations and centrifugally concentrated frozen preparations.

Furthermore, in order that the gene may be easily localized in the neighborhood of the affected site, a sustained-release preparation (eg. a minipellet) may be prepared and implanted near the affected site or the drug may be administered continuously and gradually to the affected site by means of an osmotic pump or the like.

The polynucleotide content of the drug for gene therapy can be judiciously adjusted according to the disease to be treated, the patient's age and body weight, and other factors but the usual dosage in terms of each polynucleotide is about 0.0001.about.
10 about 100 mg. preferably about 0.001.about. about 10 mg. This amount is preferably administered several days or a few months apart.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or
15 step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a
20 context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.
25 The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

Experimental Details

30 Materials and Methods

Reagents and Antibodies

Cytochalasin D, forskolin, 6-Methoxyquinolinium 1-acetic acid ethyl ester (MQAE),
35 nocodazole, 3'3'5'5' Tetra methyl benzidine, 1,4-diazabicyclo[2.2.2.]octane (DABCO), poly-D-lysine and 1% collagen were purchased from Sigma (St. Louis, MO,

U.S.A.). Lipofectin reagent and antisense oligonucleotides were purchased from Invitrogen (Mulgrave, Vic, Australia). Jasplakinolide was purchased from Bio Scientific (Gymea, N.S.W., Australia). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (NBT and BCIP), tissue culture medium and reagents were purchased from Life Technologies (Mulgrave, Vic, Australia). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford IL, U.S.A.). Thermanox coverslips and glass chamber slides were purchased from Medos (Mt Waverley, Vic, Australia). Tissue culture plasticware was purchased from Interpath (Morrisville North Carolina, U.S.A.). Western Lightening™ chemiluminescence reagent was purchased from Perkin Elmer Life Sciences Inc (Boston, MA, U.S.A.).

Rhodamine Red X conjugate and rhodamine goat anti-sheep IgG were from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Horse radish peroxidase (HRP) anti-mouse and anti-rabbit IgG were from Amersham Life Sciences (Buckinghamshire, U.K.). The mouse monoclonal Tm antibodies 311 and the secondary antibody fluorescein isothiocyanate (FITC)-Donkey anti-mouse were from Sigma Aldrich (St. Louis, MO, U.S.A.). Tm antibody CG3 was a gift from J.C. Lin (Univ. of Iowa, Iowa, U.S.A.). CFTR antibody (MA1-935) was from Affinity Bioreagents Inc. (Golden, CO, USA). The mouse monoclonal anti-human, c-terminus specific, CFTR antibody was from Bio Scientific (Gymea, N.S.W., Australia).

Cell culture

Human T84 colonic carcinoma cells were seeded onto 2 chamber glass slides, 24 or 96 well plates or glass coverslips coated with poly-D-lysine and 1% collagen. The T84 cells were obtained from the American Tissue Culture Laboratory (passage 60) and as a kind gift from Kim Barrett (San Diego, U.S.A.) (passage 20). During the course of the research, they were subcultured to passage 80 and 30 respectively. T84 cells were cultured using the method of Li *et al* (Li *et al.*, 1999, Infection & Immunity 67, 5938-5945).

The viability of T84 cells following treatments was assessed using a trypan blue exclusion assay. Post-treatment, the T84 cell monolayers were washed gently with PBS and stained with 1% trypan blue for 10 minutes. The cells were examined immediately by phase contrast microscopy. Treated and control monolayers were

compared by counting the number of cells with trypan blue uptake in random microscopy fields.

Immunofluorescence analysis

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- Cells were washed in 2% foetal bovine serum (FBS) in phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde. They were permeabilised with 100% methanol, chilled to -80°C, for 20 minutes. Cells were incubated at room temperature with primary and secondary antibodies for 1 hour with washes performed with 2% FBS
10 in PBS 3 times for 10 minutes after each incubation. Coverslips were mounted onto the slides with the anti-fade reagent DABCO.

Fluorescence microscopy

- 15 Fluorescence was examined with a confocal laser scanning microscope (Leica Microsystems, Wetzler, Germany) using a 63x oil emersion objective. The distribution of fluorophores was measured by scanning at 488 nm for FITC and 568 nm for rhodamine using 8 line averages to eliminate noise. Images were taken in the vertical (xz) and horizontal (xy) plane. Images in the horizontal plane were constructed by
20 overlaying sections taken at 1 μ m steps from the apical to the basal region of the cells.

The pixel intensity of Tm antibody staining was measured on images obtained by confocal microscopy. Measurements were made across the apical region and across the central region of monolayers and averaged to obtain the mean pixel intensity for
25 individual monolayers. The distribution of antibody staining within individual monolayers is described as the ratio of the mean pixel intensity in the apical region to the mean pixel intensity in the central region of that monolayer. To determine the relative distribution of α f9d and 311 antibody staining, the apical:central mean pixel intensity ratios for α f9d and 311 were compared in co-stained monolayers, using
30 Student t-test for paired samples.

Antibody staining of histological specimens

- Rat duodenal tissue specimens were fixed in 4% paraformaldehyde saline and stored in
35 70% ethanol at 4°C until embedded in paraffin. Sections were dewaxed in xylol and rehydrated stepwise in graded ethanols (100%, 100%, 70%, water). Antigen retrieval

was performed by boiling the specimens in 1x citrate buffer (10x citrate buffer: 5g/l EDTA, 2.5g /l Tris base and 3.2 g/l tri-sodium citrate; pH 8.0), microwaving on high for 12 minutes then allowing to cool. The specimens were washed twice in PBS and blocked with 10% serum in PBS for 10 minutes. Primary antibodies were then applied

- 5 overnight at room temperature. Specimens were washed twice in PBS for 5 minutes prior to application of the secondary antibodies. Secondary antibodies were applied for 1 hour after which the specimens were washed once in PBS for 5 minutes and once with alkaline phosphate buffer (10 mls of 0.1M tris pH 9.5, 5 mls 1M MgCl and 2 mls of 5M NaCl) for 5 minutes. The substrate containing NBT and BCIP was then applied
- 10 for 40 to 60 minutes after which specimens were washed once with PBS for 5 minutes. Specimens were then counterstained with Nuclear Fast Red for 1 minute after which they were rinsed twice in distilled water, dehydrated in increasing grades of ethanol (70%, 100%, 100%, 100%), cleared with xylol and coverslipped.
- 15 *Cell treatment with jasplakinolide, cytochalasin and nocodazole during monlayer formation*

- Epithelial cell monolayers were trypsinised using trypsin/EDTA and centrifuged to form a cell pellet. The cells were then resuspended in medium containing either 1 μ M
- 20 jasplakinolide, 20 μ M cytochalasin D or 33 μ M nocodazole and seeded into poly-d-lysine and collagen coated glass chamber slides. The developing monolayers were then fixed and stained at 10 minutes after seeding. The effect of nocodazole on microtubules was confirmed by staining with antibodies to β tubulin and comparing with untreated cells. Mature T84 cell monolayers were treated with medium containing 20 μ M
 - 25 cytochalasin D for 3 hours then fixed and stained. Immunofluorescence analysis was then performed as described above.

Antisense Oligonucleotides

- 30 The sequence of the antisense and nonsense phosphorothioated oligonucleotides to Tm5a and Tm5b were 5'- CAC CGC CUC CAG CGA GCT and 5'- GCT CCA GCC ACG CCG ACT respectively. These were designed from the exon 1b sequence of the human α TMfast gene (Novy *et al.*, 1993, Cell Motility & the Cytoskeleton 25, 267-281). T84 cell monolayers were grown to confluence on coverslips, in glass chamber slides or 24 well plates. The oligonucleotides were applied at a concentration of 2 μ M with Lipofectin Reagent at 10 μ g/ml according to the manufacturers instructions. The
- 35

T84 cell monolayers were then incubated with the oligonucleotide for 24 hours at 37°C in 5% CO₂ after which time they were used for experiments that required oligonucleotide pretreatment.

5 *Immunoblot analysis of tropomyosin isoforms*

- Proteins were extracted from T84 cells using the method of Wessel and Flugge (Wessel and Flugge, 1984). Western blot was performed as described (Percival *et al.*, 2000, Cell Motility & the Cytoskeleton 47, 189-208). In brief, proteins were fractionated by 10 SDS-PAGE using 15% low bis acrylamide gels, transferred to polyvinylidene difluoride membranes and probed with Tm antibodies. Bound antibody was detected using HRP-conjugated goat anti-rabbit or goat anti-mouse IgG. The bands were detected using Western Lightening™ chemiluminescence reagent and exposure to x-ray film.
- 15 Protein expression was measured as the density of protein bands on Western blot autoradiographs using the computer program Molecular Analyst (Version 1.5, Bio Rad Laboratories, CA, U.S.A.). The protein band density is reported as the protein band density normalised to the protein band density for the control group within individual 20 experiments. To determine the effects of treatment, normalised protein band density was compared to the null hypothesis value of 1 by the one-sided Student t test.

MQAE chloride efflux assay

- 25 T84 cell monolayers, cultured on 24 or 96 well plates were incubated in medium containing 10mM MQAE for 16 hours. The monolayers were then washed 3 times in chloride buffer (2.4mM Na₂HPO₄, 0.6mM NaH₂PO₄, 1mM K₂SO₄, 1mM MgSO₄, 3.4mM KCl, 124.6mM NaCl, 1mM CaCl₂, 10mM glucose and 10mM HEPES). T84 cell monolayers were stimulated with forskolin by incubating with chloride buffer 30 containing 10μM forskolin for 10 minutes, after which the chloride buffer was removed and replaced with chloride free buffer (2.4mM Na₂HPO₄, 0.6mM NaH₂PO₄, 1mM K₂SO₄, 1mM MgSO₄, 3.4mM KNO₃, 1mM Ca(NO₃)₂, 124.6mM NaNO₃, 10mM glucose and 10mM HEPES) containing 10μM forskolin. Repetitive fluorescence measurements were initiated immediately using a fluorescence plate reader (excitation, 35 λ-360nm; emission, λ-460nm). Measurements were performed every 30 to 60 seconds for 15 minutes.

Chloride efflux was measured as the percentage increase in fluorescence between baseline and the specified time point. The percentage increase in fluorescence was normalised within experiments to the mean percentage increase in fluorescence in the 5 control group in that experiment. To determine the effects of treatments, the normalised percentage increase in fluorescence was compared between groups. Two group comparisons were made using the Student t test.

Enzyme linked surface CFTR assay

- 10 T84 cell monolayers cultured on collagen coated glass coverslips were incubated in either chloride buffer with 10 µM forskolin or chloride buffer only for 30 minutes at 37°C in 5% CO₂ then fixed with 4% paraformaldehyde for 20 minutes at 4°C. The T84 cell monolayers were incubated for 1 hour firstly with CFTR (MA1-935) antibody 15 (Walker *et al.*, 1995) diluted 1:500 followed by HRP anti-mouse IgG diluted 1: 1000. T84 cell monolayers were blocked prior to each incubation for 10 minutes in PBS containing 10% FBS and washed following each incubation 4 times in PBS. The coverslips were then placed into a clean 24 well plate and incubated for 30 minutes with 500 µl of 3'3'5'5' Tetra methyl benzidine. The supernatant from each well was 20 transferred to a cuvette and absorbance determined at 655 nm in a Beckman DU650 spectrophotometer. Absorbance was also determined at 655nm for primary antibody negative controls and that amount was subtracted from the absorbance in primary antibody positive monolayers to determine their assay result.
- 25 The CFTR surface expression is reported as the absorbance measured at 655nm, normalised to the mean absorbance for the control group within individual experiments. To determine the effects of treatment, normalised absorbance at 655nm was compared between groups. Two group comparisons were made using the Student t test.
- 30 **Example 1: Tropomyosin gene expression and antibody specificity in T84 cells**

- Tm proteins are encoded by 4 distinct genes. The antibodies used in this study were capable of detecting specific isoforms that are produced from 3 Tm genes. The exon/intron structure of these genes is shown in Figure 1. The αf9d antibody detects 35 Tm 1, 2, 3, 5a, 5b and 6 (Schevzov *et al.*, 1997, Molecular & Cellular Neurosciences 8, 439-454). The 311 antibody detects a subset of Tms detected by the αf9d antibody,

namely Tm 1, 2, 3, and 6. The CG3 antibody detects Tm5NM1-11 (Novy *et al.*, 1993, Cell Motility & the Cytoskeleton 25, 267-281; Dufour *et al.*, 1998, Journal of Biological Chemistry 273, 18547-18555).

- 5 In human fibroblasts, the 311 antibody detected 3 bands. Bands were seen at 40, 36 and 34 kDa corresponding to Tm 6, 2 and 3 respectively (Figure 2A). In T84 cells, the 311 antibody detected only the bands at 40 and 34 kDa corresponding to Tm 6 and 3 (Figure 2A). The α f9d antibody detected 4 bands in T84 cells with bands seen at 40 and 34 kDa corresponding to Tm6 and Tm3 and a double band at 30 kDa 10 corresponding to Tm's 5a and 5b (Figure 2B). The CG3 antibody detected a single band at 30 kDa corresponding to co-migrating Tm5NM isoforms (Figure 2C).

Example 2: T84 cell monolayers express a polarised distribution of Tm5a and Tm5b

- 15 To determine the distribution of the separate microfilament populations in T84 cells, eight monolayers stained with each antibody were examined in both the vertical and horizontal planes by confocal microscopy. Representative images are presented in Figure 3. The α f9d antibody, which detects Tm 3, 5a, 5b and 6 was found to have 20 predominant staining at the apical pole of the cells (Figure 3A). However, the 311 antibody, which recognises Tm 3 and 6, was found to have a more uniform distribution from the apical to basal pole of the same cells (Figure 3C). This differential staining pattern can only be explained by the existence of a highly polarised distribution of the two isoforms detected by α f9d which are not detected by 311 (i.e. Tm5a and Tm5b). 25 We therefore conclude that Tm5a and Tm5b are highly enriched at the apical surface. The antibody CG3, which stains Tm5NM 1-11, was distributed throughout the cell (Figure 3E).

In sections through the epithelial monolayer taken in the horizontal plane, the 30 distribution of α f9d (Figure 3B) and 311 (Figure 3D) were found to be associated with the lateral cell membrane and a paucity of staining was seen in the cytoplasm. CG3 was found to be located in the cytoplasm surrounding the cell nucleus (Figure 3F).

The quantitative analysis of the relative distribution of α f9d and 311 antibody staining, 35 which is depicted in Figure 3G, confirmed the qualitative differences described above. The mean ratio of apical to central pixel intensity for the α f9d antibody was

significantly higher than that of the 311 antibody (3.88 ± 0.60 vs 1.64 ± 0.23 ; $p < 0.001$).

Example 3: The polarised distribution of specific microfilament populations varies with epithelial cell differentiation in the rat duodenum.

To determine whether the distribution of Tm isoforms observed in T84 cells differed from that seen *in vivo* in both crypt and villus gastrointestinal epithelial cells, 6 rat duodenal tissue specimens were stained for Tm isoforms and examined with brightfield microscopy. Representative sections are shown in Figure 4. Staining with the α f9d antibody showed diffuse staining in the crypt epithelium (Figure 4C arrow). The staining in the more differentiated villus epithelium was highly enriched in the apical region but also seen throughout the cytoplasm (Figure 4D arrow). Staining with the 311 antibody (Tm 3 and 6) was sparse in the crypt epithelium (Figure 4E). In the villus epithelium, the blue staining was seen in a circular area located above the nucleus (Figure 4F). Staining with the CG3 antibody (Tm5NM 1-11) showed a similar distribution to that seen with the α f9d antibody. In the crypt epithelial cells, the staining was diffuse throughout the cell (Figure 4G), whereas in the villus epithelial cells there was strong enrichment of staining in the apical region (Figure 4H). In the goblet cells, which are predominantly found in the crypts, the staining was diffuse outside of the characteristic mucinous vacuole (Figure 4G).

These results demonstrate that Tm isoforms are polarised in the more differentiated villus epithelial cells and are not polarised in the less differentiated crypt epithelial cells. Importantly, the relative distributions of α f9d antibody and 311 antibody staining in duodenal villus epithelial cells indicates that Tm5a and Tm5b are polarised *in vivo* in the same way they are polarised in the T84 cell model.

Example 4: Polarised distribution of specific microfilament populations occurs in the early phases of monolayer formation

The time sequence over which the polarised distribution of α f9d staining occurs was examined at 10 minutes, 1, 2 and 24 hours after seeding T84 cells. Three experiments were performed for each time point. Tm isoform expression was examined by performing Western blots on protein extracted from T84 cells collected at 1, 2, 4 and 24 hours and 7 days post seeding. Three experiments were performed at each time point.

Representative confocal microscopy images are shown in Figure 5. In T84 cells seen in suspension, α f9d, 311 and CG3 (Figure 6A, 6B and 5I, arrow and data not shown) antibody staining was circumferential. Ten minutes after seeding (5A-C), the T84 cells 5 were generally observed to make cell-cell contact and cell-slide contact. For all antibodies, there was reduced staining at the site of cell-slide contact at this initial time point although staining is more apparent for CG3 and 311 than for α f9d. Further, α f9d antibody staining appeared to be limited to the free surface while 311 antibody staining 10 was more prominent at the site of cell-cell contact. In contrast, CG3 antibody staining was more evenly distributed over both the free surface and sites of cell-cell contact. Over time, the distribution of α f9d staining was basically unchanged (5 E, H and K) 15 with enriched staining at the free surface (reflecting Tm5a and Tm5b) and lower level diffuse staining resembling that of 311 (reflecting Tm6 and Tm3). In contrast, the distribution of 311 antibody staining (5 D, G and J) and CG3 antibody staining (5 F, I and L) both became more evenly distributed throughout the cell to include all surfaces and the cytoplasm.

Western blot analysis revealed that T84 cells collected 2 and 4 hours post seeding had a slightly increased expression of Tm 6 and 5a compared with cells collected at 24 hours 20 and 7 days post seeding (Figure 5N). The changes in levels of these isoforms cannot account for the alterations in staining of the α f9d and 311 antibodies. These alterations in isoform distribution are therefore most likely to result from altered targeting of these proteins.

25 Example 5: The early polarised distribution of Tm5a and Tm5b does not involve filament turnover and is not microtubule dependent

Possible mechanisms for the development of microfilament polarisation were explored by drug manipulation of the cytoskeleton during seeding. Jasplakinolide was used to 30 stabilise actin filaments, cytochalasin D was used to fragment actin filaments and nocodazole was used to disrupt microtubules. These drugs were applied to the T84 cells while they were in suspension, 10 minutes prior to plating. Cells were examined 10 minutes after plating.

35 Pretreatment of T84 cells with jasplakinolide prior to seeding altered cell morphology. The T84 cells had a flattened appearance (Figure 6A) compared with untreated cells

(Figure 5A). In the T84 cells pretreated with jasplakinolide, the distribution of both $\alpha f9d$ (Figure 6B) and 311 (Figure 6A) antibody staining 10 minutes post-seeding was similar to that of the control T84 cells (5A and 5B). The distribution of $\alpha f9d$ antibody remained apical, whilst the 311 antibody distribution appeared more prominent at the sites of cell-cell contact. Pretreatment of T84 cells with cytochalasin D prior to seeding prevented cell-slide adherence and no images were obtained. However, treatment of established monolayers with cytochalasin D eliminated the polarised distribution of $\alpha f9d$ staining indicating that its maintenance requires an intact actin cytoskeleton (Figure 6F)

10

- Pretreatment of the T84 cells with nocodazole prior to seeding altered cell morphology. The T84 cells changed from having a curved surface (Figure 5A) to having an irregular appearance (Figure 6C). The distribution of the $\alpha f9d$ (Figure 6D) antibody staining in nocodazole treated T84 cells 10 minutes post-seeding was similar to that of untreated T84 cells. Staining with the 311 antibody appeared similar to that of the $\alpha f9d$ antibody with enrichment at the apical surface and paucity of staining at the site of cell contact with the slide (Figure 6C). Staining for β tubulin confirmed that nocodazole had disrupted normal microtubular structure (Data not shown).
- 15 These results suggest that the early polarisation of Tm5a and Tm5b does not involve filament turnover because the actin stabilising agent jasplakinolide did not affect the early development of polarisation. In addition, intact microtubules are not required as polarisation of Tm5a and Tm5b occurred despite microtubular disruption with nocodazole. However, microtubules may be involved in relocation of Tm3 and Tm6 to sites of cell-cell contact or their stabilisation at that site.
- 20

Example 6: Tm5a and Tm5b co-localise with membrane inserted CFTR but not CFTR contained in sub-apical vesicles

- .30 Staining of T84 cells with the CFTR antibody (Figure 7B) demonstrated variable expression of CFTR. CFTR was seen in two forms. Some cells demonstrated prominent apical staining, with the CFTR appearing to protrude at the apical membrane. CFTR was also seen as smaller dots (Figure 7B, arrow) located in the cell cytoplasm, giving the appearance of a location within a vesicle-like structure. Co-35 staining with the $\alpha f9d$ antibody revealed the typical polarised appearance at apical enrichment in addition to sites of very intense apical staining projecting above the

surrounding apical surface. These highly enriched sites of α f9d staining were coincident with the sites of membrane incorporation of CFTR (Figure 7C). The Tms therefore appeared to be incorporated into a structure associated with CFTR. All sites of membrane staining of CFTR were associated with these intense sites of α f9d staining. However, not all intense sites of α f9d staining showed significant CFTR staining suggesting that α f9d antibody staining is associated with sites available for CFTR insertion. The α f9d antibody did not co-localise with the CFTR contained within the cytoplasmic vesicle-like structures.

10 **Example 7: Tm5a and Tm5b antisense oligonucleotides alter the intensity of apical staining of α f9d in T84 cell monolayers**

Previous studies have shown that cytochalasin D induced disruption of actin filaments increase chloride currents through CFTR (Prat *et al.*, 1995, American Journal of Physiology 268, C1552-C1561) and we have observed that cytochalasin D also disrupts the polarised distribution of α f9d staining (Figure 6F). We therefore reasoned that the actin filaments marked by α f9d, which colocalise with CFTR might inhibit chloride secretion by CFTR. To test this, we treated T84 cell monolayers with an antisense oligonucleotide or a scrambled nonsense control. Western blot analysis showed a substantial reduction in Tm5a and Tm5b levels after 24 hours exposure to the oligonucleotide (Figure 8D). Relative to nonsense treatment, the antisense produced a mean reduction in Tm5a and Tm5b levels of $54 \pm 13\%$ ($p=0.02$).

The treatment of T84 cultures with antisense oligonucleotide eliminated the polarised distribution of α f9d staining, which became largely even throughout the cell (Figure 8B). In contrast, the nonsense oligonucleotide had essentially no effect on the distribution of staining with α f9d (Figure 8A). The redistribution of staining induced by the antisense oligonucleotide was paralleled by a reduction in pixel intensity of α f9d staining at the apical surface. T84 cell monolayers treated in parallel with these oligonucleotides resulted in less apical pixel intensity in antisense cultures compared with nonsense cultures (Figure 8E). This is consistent with a decrease in the level of polarised Tms detected by the α f9d antibody.

In conclusion, treatment with an antisense oligonucleotide to exon 1b of the α fast gene resulted in a significant reduction in apical staining with the α f9d antibody. These

results also confirm that the prominent apical α f9d antibody staining in untreated T84 cell monolayers is due to a polarised distribution of Tm5a and Tm5b.

Example 8: Tm5a and Tm5b antisense oligonucleotides increase CFTR surface expression and chloride efflux

Antisense reductions of Tm5a and Tm5b levels and elimination of the polarised distribution of α f9d staining provided the opportunity to assess the role of these molecules in CFTR surface expression. This revealed a 50% increase in antisense 10 compared with nonsense controls (1.49 ± 0.78 vs 1 ± 0.42 ; $p < 0.001$). This suggests that the presence of Tm5a and Tm5b is acting either as a barrier to CFTR insertion into the apical membrane or retention of CFTR in the membrane.

The increase in CFTR surface expression was paralleled by an increase in chloride 15 efflux from antisense treated cells. In total, 21 T84 cell monolayers were treated with $2\mu\text{M}$ antisense for 24 hours and were compared with 21 T84 cell monolayers treated with $2\mu\text{M}$ nonsense for 24 hours. Following antisense and nonsense treatment, an MQAE chloride efflux assay was performed. The results are depicted in Figure 9B. The T84 cell monolayers treated with antisense had significantly higher relative 20 fluorescence measurement than T84 cell monolayers treated with nonsense after 15 minutes of $10\mu\text{M}$ forskolin stimulation (1.47 ± 0.41 vs 1 ± 0.36 ; $p < 0.001$).

Example 9: Microtubule disruption has no effect on CFTR surface expression in T84 cell monolayers

25 The impact on CFTR surface levels and chloride efflux by antisense treatment of Tms was not paralleled by disruption of microtubules. Incubation of T84 cells with nocodazole failed to elicit any significant change in either CFTR surface expression (Figure 10A) nor chloride efflux (Figure 10B). We conclude that these parameters are 30 sensitive to disruption of the microfilament but not microtubule systems when assayed under short-term conditions. This correlates well with a more important role for actin filaments rather than microtubules in regulating the insertion of vesicle cargo's into the apical membrane or their retention.

Example 10: The effect of Enteropathogenic *E. coli* (EPEC) infection on the actin cytoskeleton

Enteropathogenic *E. coli* (EPEC) is responsible for up to 17% of gastroenteritis in children from Australian aboriginal communities. The mechanism by which it causes diarrhoea is unclear but increased chloride secretion has been implicated in animal models. We have previously demonstrated, in a cell culture model, that EPEC infection causes a reduction in epithelial cell chloride secretion through CFTR chloride channels and induces a redistribution of tropomyosin 5a and 5b isoforms within the epithelial cell's cytoskeleton. The function of these tropomyosins is unknown, but we have demonstrated that they are co-localised with CFTR chloride channels in the apical membrane.

The aim of this experiment was to examine the mechanism by which EPEC infection alters chloride secretion through CFTR chloride channels.

Cultured T84 colon cancer cell monolayers grown in collagen coated 24 well plates or on plastic coverslips were used as a model of the gastrointestinal epithelium. Incubation of monolayers inoculated with EPEC (104 organisms/well) for 6 – 9 hours was used to model EPEC infection and was compared with HB101, a non-pathogenic control. Antisense oligonucleotides were used to reduce tropomyosin 5a and 5b expression. An immuno-colourimetric assay was used to assess CFTR surface expression and intracellular MQAE fluorescence was used to assess chloride efflux.

25 The results showed that CFTR expression was increased (Mean increase: 153%; 95% CI: 100%, 205%; p < 0.001) but chloride secretion was decreased by EPEC infection compared with HB101 (Mean decrease: 37%; 95% CI: 8%, 66%; p=0.014).

30 The redistribution of tropomyosin 5a and 5b may be causally related to the increased CFTR insertion in the apical membrane found with EPEC infection. Tropomyosin 5a and 5b containing filaments may provide a barrier to insertion of CFTR or retention in the apical membrane of gastrointestinal epithelial cells. The decrease in chloride secretion in the presence of elevated membrane CFTR suggests that EPEC can also inhibit CFTR chloride channel function. Diarrhoea may occur in EPEC infection as a 35 rebound phenomenon following recovery of CFTR function in the presence of increased surface expression.

These results suggest that EPEC contains a compound that is capable of inhibiting the location or function of TM5a and TM5b. EPEC may therefore be a useful source of material for use in the screening assays described herein.

5

Discussion

Tropomyosin isoform sorting in establishing epithelial cell polarity

- 10 The development of polarisation with the creation of specialised functional domains is necessary for normal epithelial cell function. Central to the process of epithelial cell polarisation is the sorting, transport and insertion into the membrane of proteins, which give the domains their function (Yeaman *et al.*, 1999, Physiological Reviews 79, 73-98). The role of the cell cytoskeleton, in particular the actin microfilament system, in this process is not clear. The rapid generation of actin cytoskeletal domains raises the possibility that cytoskeletal polarisation may be required for the development of functional polarity, particularly sorting and movement of proteins to specific membrane domains.
- 15
- 20 The findings in this study further support a role for microfilaments in the development of epithelial cell polarity and the polarised delivery of membrane proteins. We found that specific Tm isoforms polarise rapidly during monolayer development. The same isoforms were also found to regulate the insertion of CFTR and/or its retention in the apical membrane.

25

Mechanisms of isoform sorting

- Drugs that interact with the cytoskeleton are widely used to examine cellular processes. By using these techniques, we were able to examine the mechanism by which epithelial cells were rapidly able to sort microfilaments. In the developing monolayer Jasplakinolide, a drug that prevents the breakdown and turnover of actin filaments, did not affect the early polarisation of Tm5a and Tm5b. In mature monolayers cytochalasin D, which breaks up actin filaments, disrupted the polarised distribution of Tm5a and Tm5b. Thus we can conclude that intact microfilaments are required for both the development of polarisation of Tm isoforms as well as the maintenance of this
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polarity. In addition the Tm isoforms form part of a higher order structure involving actin filaments rather than existing as isolated molecules.

The sorting of Tm isoforms found in our study occurred very rapidly. Within 10 minutes, specific Tm isoforms became polarised in their distribution. Other investigators have also found that changes in Tm and actin structure and composition occurs early in the development of cell structure. In a study by Temm-Grove *et al*, specific Tm isoform localisation occurred as soon as 15 minutes after being micro-injected into an epithelial cell (Temm-Grove *et al.*, 1998, *Cell Motility & the Cytoskeleton* 40, 393-407). They found that Tm5 localised rapidly to the adhesion belt between adjacent cells. Other studies have examined expression levels with time. In fibroblasts, Tm 5NM isoform expression level increased 2-fold by 5 hours during the cell cycle (Percival *et al.*, 2000, *Cell Motility & the Cytoskeleton* 47, 189-208). In cultured hepatocytes, F actin mass increased 20 fold within 30 minutes of cell adhesion to extra-cellular matrix (Mooney *et al.*, 1995, *Journal of Cell Science* 108, 2311-2320). In developing neurones, Tm5 mRNA was found to localise to the axonal hillock thus forming an early marker of neuronal polarity (Hannan *et al.*, 1995, *Molecular & Cellular Neurosciences* 6, 397-412). Thus we conclude that cells of various types are capable of rapidly altering their cytoskeletal structure either by increasing cytoskeletal protein expression or moving intact protein within the cell. These findings implicate Tm in the early processes of cell attachment and the development of polarisation.

Role of Tm5a and Tm5b on regulating CFTR function

Without wishing to be bound by theory, there are at least three possible mechanisms which may explain how Tm5a and Tm5b limit CFTR insertion into the apical membrane in response to cAMP stimulation. Firstly, that Tm5a and Tm5b may act as a physical barrier to vesicle movement towards the apical surface of the epithelial cell. When removed, vesicle movement would occur more freely and a subsequent increase in membrane inserted CFTR would be inevitable. Secondly, Tm5a and Tm5b may not act as a functional barrier to vesicle movement but may be an inhibitory control mechanism for the movement of vesicles along actin filaments. The movement of vesicles along actin filaments is an active process that requires the interaction of actin and myosin. Tm5a and Tm5b may inhibit this interaction. If this were the case, the presence of Tm5a and Tm5b in the apical region would be expected to inhibit the delivery of CFTR vesicles to the apical membrane. Conversely, depolarisation of

Tm_{5a} and Tm_{5b} would be expected to increase the delivery of CFTR to the apical membrane. Finally, Tm_{5a} and Tm_{5b} associated microfilaments may be involved in the process of endocytic cycling of surface proteins. A study by Gottlieb *et al* found that microfilaments play a role in the endocytosis of proteins at the apical membrane of epithelial cells (Gottlieb *et al.*, 1993, *Journal of Cell Biology* 120, 695-710). From their observations, they hypothesised that actin microfilaments form part of a mechanochemical motor that is involved in either moving microvillar membrane components towards the intervillar spaces or providing the force to convert membrane pits into endocytic vesicles. If the microfilaments involved in these processes contain Tm_{5a} and Tm_{5b}, then the removal of Tm_{5a} and Tm_{5b} would result in failure to endocytose proteins such as CFTR from the apical membrane.

Our finding that Tm_{5a} and/or Tm_{5b} regulate the insertion or retention of CFTR into the apical membrane contributes further to the growing body of evidence that Tm isoforms have different functions. There are over 40 Tm isoforms known to exist (Lees-Miller and Helfman, 1991, *Bioessays* 13, 429-437; Pittenger *et al.*, 1994, *Current Opinion in Cell Biology* 6, 96-104) (Dufour *et al.*, 1998, *Journal of Biological Chemistry* 273, 18547-18555). Supportive of the presence of differing functions is the knowledge that the various Tms confer different mechano-chemical properties to actin microfilaments. For example, the differing binding affinities of Tm isoforms for actin results in a differential effect on the stability of actin microfilaments (Pittenger *et al.*, 1994, *Current Opinion in Cell Biology* 6, 96-104). Further evidence comes from work by Percival *et al* who found that Tm_{5NM} confers greater cytochalasin D resistance to actin microfilaments (Percival *et al.*, 2000, *Cell Motility & the Cytoskeleton* 47, 189-208). Others have found that specific Tm isoforms increase the rigidity of actin filaments (Kojima *et al.*, 1994, *Proceedings of the National Academy of Sciences of the United States of America* 91, 12962-12966). Once inserted into the actin microfilament, Tms influence the interaction between actin and other actin binding proteins. For example, high molecular weight Tms are protective against the severing activity of the actin binding protein gelsolin (Ishikawa *et al.*, 1989, *Journal of Biological Chemistry* 264, 7490-7497).

Our findings contribute to a growing body of evidence supporting a role for Tms in specific cellular functions. We conclude that Tm isoforms are segregated in gastrointestinal epithelial cells and are capable of regulating important cellular functions.

All documents referred to above by reference are incorporated in their entirety into this disclosure.

- 5 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty first day of March 2003

The Royal Alexandra Hospital for Children
Patent Attorneys for the Applicant:

F B RICE & CO



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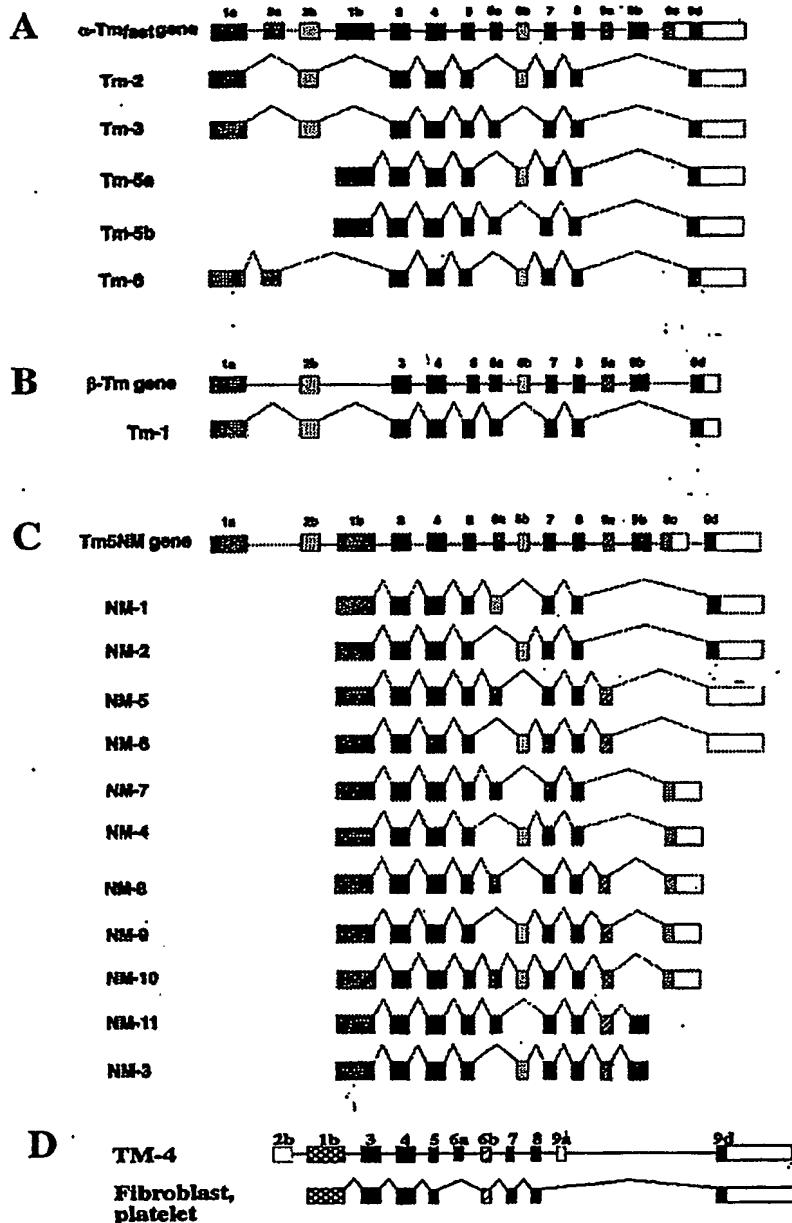


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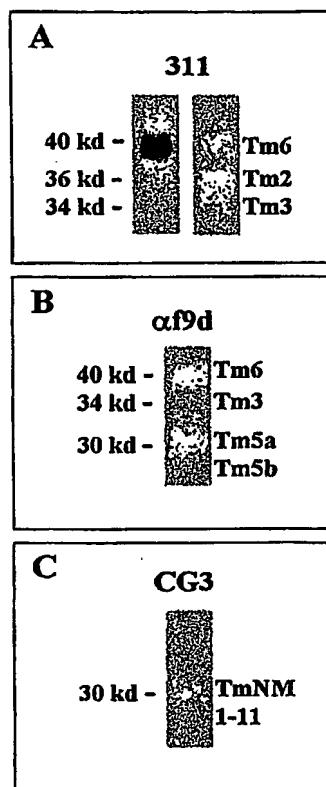
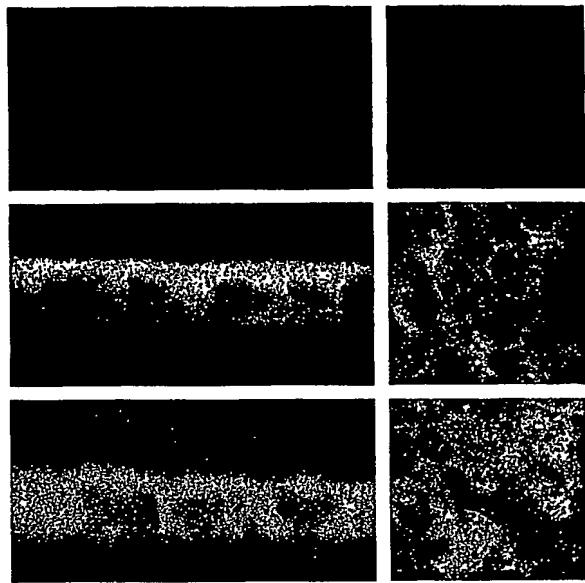


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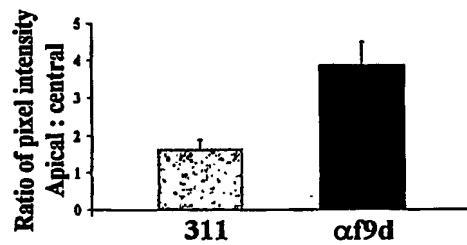


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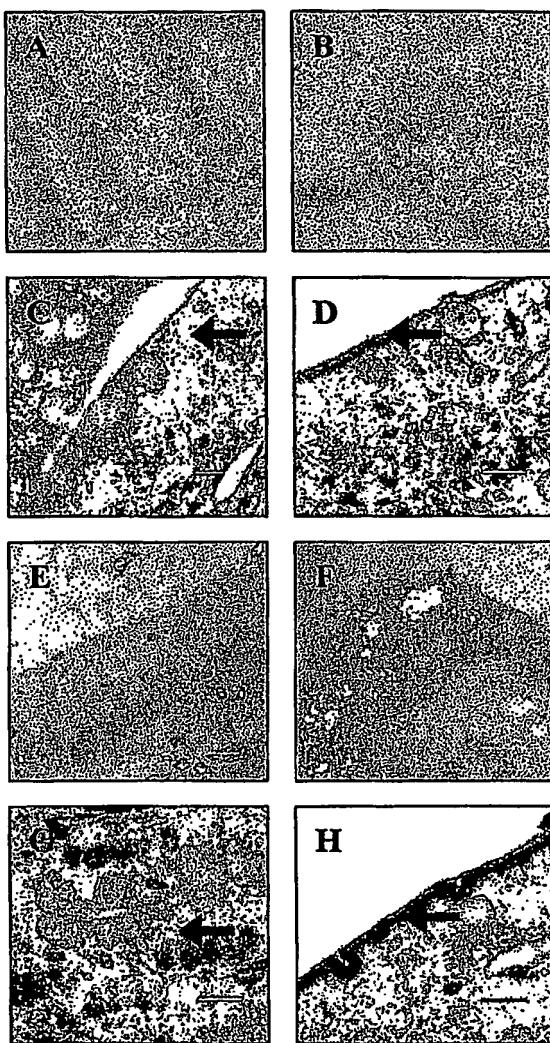


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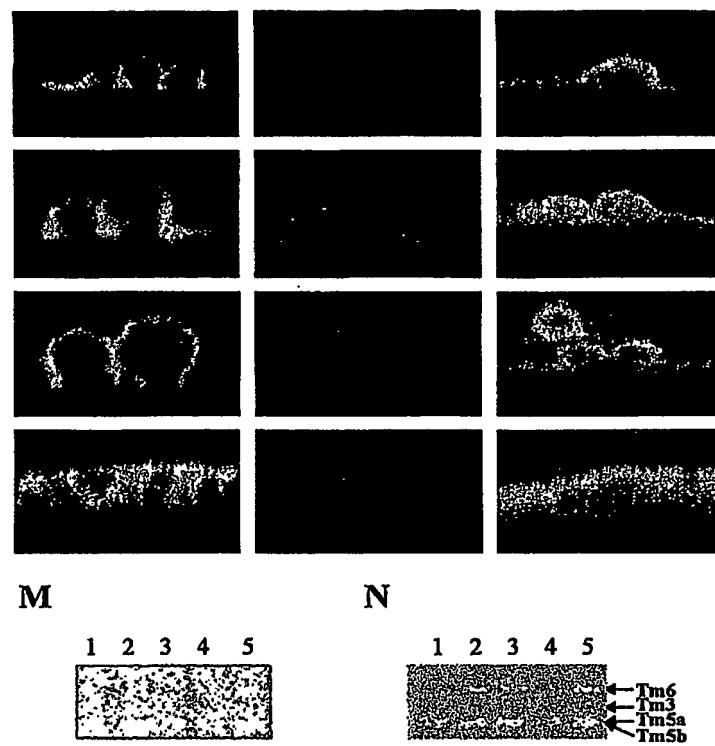


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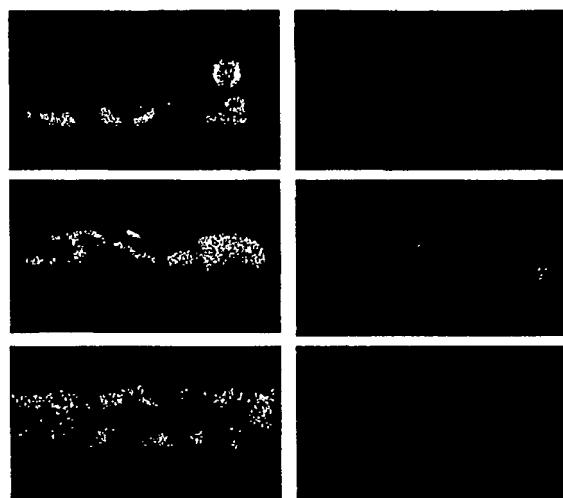


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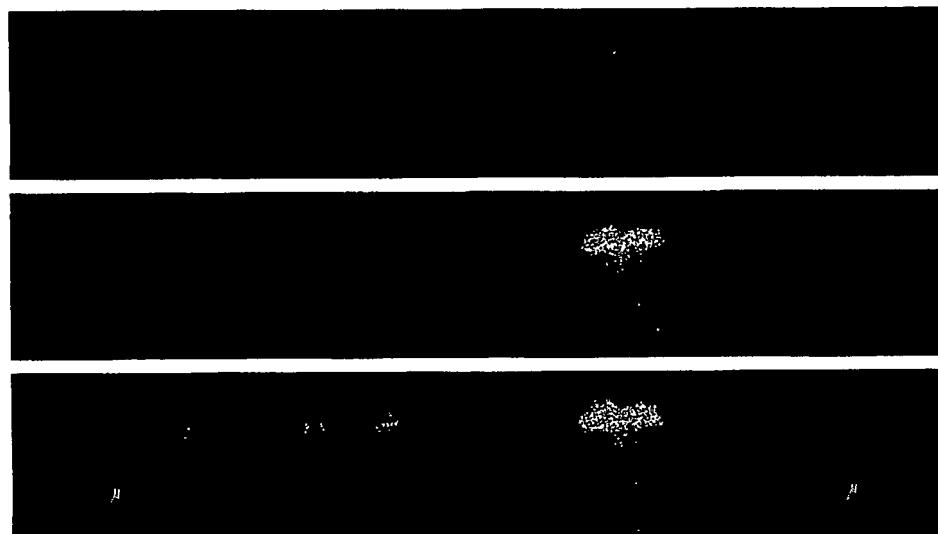


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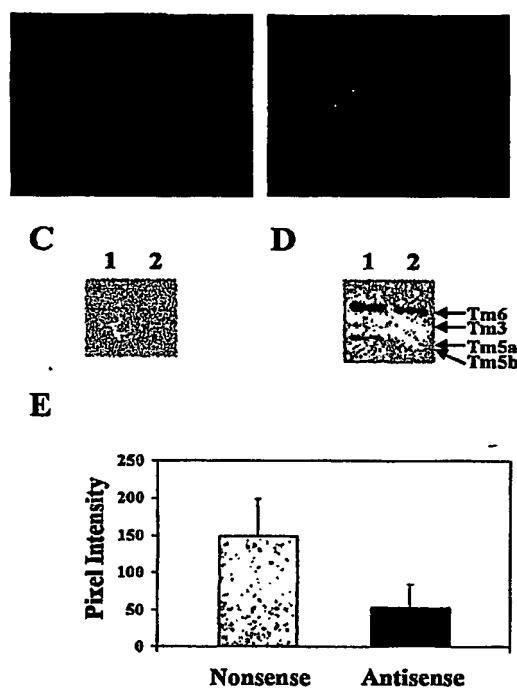


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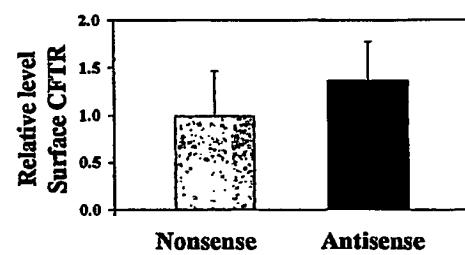
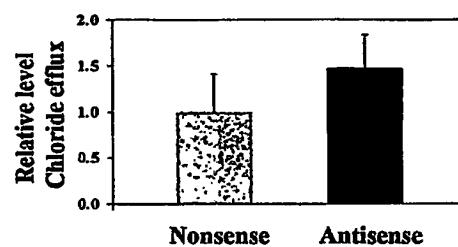
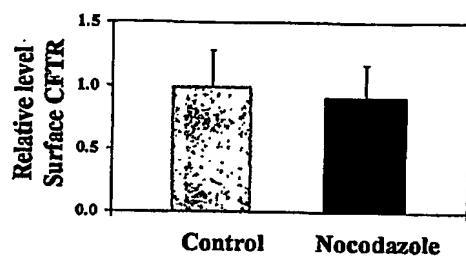
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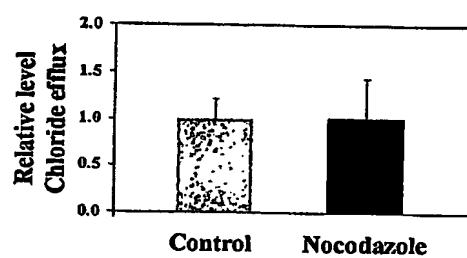


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